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THE QUARTERLY JOURNAL OF MICROSCOPICAL SCIENCE

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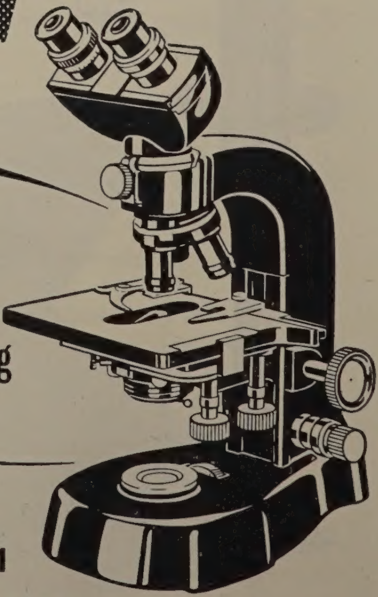

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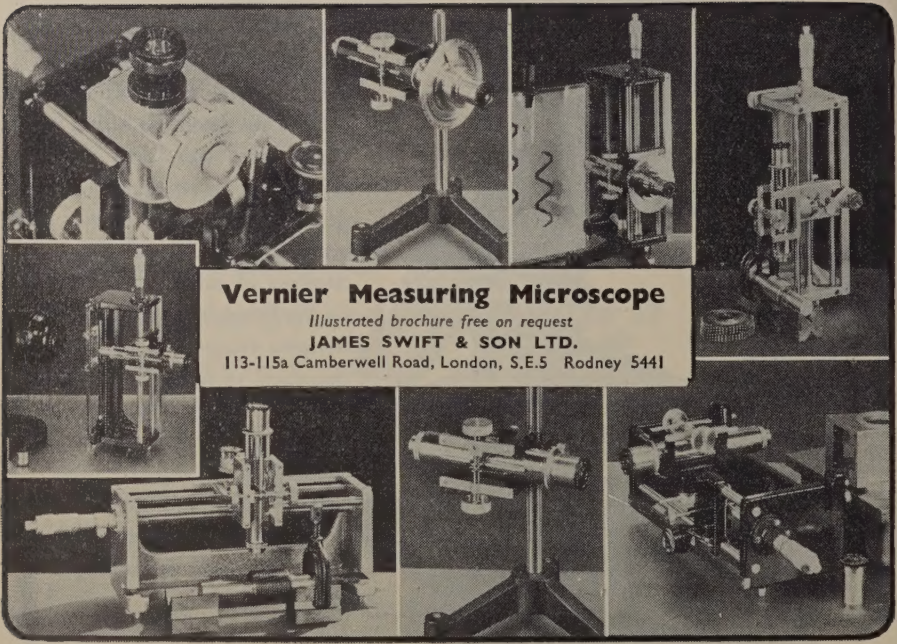

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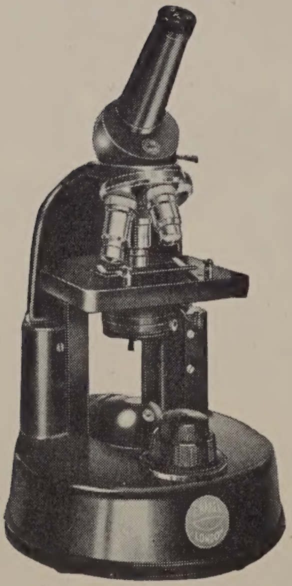

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Editors: B. C. J. G. Knight and A. F. B. Standfast

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The Nerve-net of *Metridium senile*: Artifacts and the Nerve-net

By E. J. BATHAM, C. F. A. PANTIN, and E. A. ROBSON

(*From the Zoological Laboratory, Cambridge*)

With three plates (figs. 1–3)

SUMMARY

The present paper follows an account of the structure of the nervous system of *Metridium senile* (L.). Conflicting statements about the actinian nervous system in the earlier literature made it necessary to assess the results of previous workers critically. Several of their methods have now been repeated and compared with our results after using more specific techniques. The criteria for distinguishing nerve-cells from non-nervous elements in actinians are discussed. Mesogloeal fibres, amoebocytes, nematocyst threads, and muscle-fibres may on occasion be confused with nerve-cells, and deteriorating nerve-cells may also have a misleading appearance. Gross artifacts may be reduced by the use of special staining methods, and on the basis of this work the results of several earlier authors have been re-interpreted. It is concluded that the nervous system in the mesenteries and column of *Metridium* follows the epithelial layers and does not penetrate the mesogloea.

ARTIFACTS AND THE NERVE-NET

RECENT work (Pantin, 1952; Batham, Pantin, and Robson, 1960) has emphasized the importance of exact information about the organization of the nerve-net of actinians for understanding the machinery of their elementary nervous system. Existing knowledge began with the studies of the Hertwigs of 1879. One can only be filled with admiration for the perspicacity and completeness of their work, and feel some regret that subsequent studies, upon which contemporary ideas of this sort of nervous system are based, sometimes fall short of the standards of these workers of 80 years ago.

In relating nervous organization to the activities displayed by actinians there is one major problem. According to the Hertwigs the nerve-net is essentially intra-epithelial, being confined to the two layers of ectoderm and endoderm (Hertwigs, 1879). They found some connexion between the ectodermal and endodermal nets only where the two layers join at the base of the pharynx. There may possibly be other connexions of the same sort, however, as at the pore-like cinclides that pierce the column of some anemones and pores at the tips of the tentacles or in the foot in others. Our own studies indicate that in localized places on the oral disk the mesogloea can be seen to be interrupted, so that the ectoderm and endoderm occasionally meet directly; but this work will be reported later. We find that all such contacts between the ectodermal and endodermal nervous system of actinians are limited; and the same is true of the nervous system of *Hydra* according to the very thorough study of Hadži (1909).

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The separation of nerve-nets in different epithelia not only holds between ectoderm and endoderm but also between the two sheets of endoderm that are on each side of the mesogloea of the mesenteries. This accords with the essentially separate functional action of the muscle-sheets on the two sides of the mesogloea; as in the endodermal radial and retractor muscle-sheets of the mesenteries (Batham and Pantin, 1950), and in the muscle-sheets of the ectoderm and endoderm of the tentacles and disk in actinians and in the whole body of *Hydra* (Batham and Pantin, 1951).

The conclusion of the Hertwigs that the nerve-nets are intra-epithelial is supported by that of Groselj (1909) and by our own (Pantin, 1952; Batham, Pantin, and Robson, 1960).

In sharp contrast to this picture of the organization of the nerve-net is that derived from the studies of Havet (1901), and of Parker and Titus (1916) on *Metridium*, and those of Leghissa (1949, 1950). According to all these authors, there is a very well-developed system of nerve-fibres in the mesogloea—where the Hertwigs, Groselj, and we ourselves find none. This mesogloeal system is said to supply both the ectoderm and endoderm and so would represent the primary nervous system. According to these authors the nervous system is therefore richer in nerve-cells than if it were restricted to the epithelial sense-cells and nerve-cells described by the Hertwigs, since the latter would represent only the peripheral ramifications of a complex network based on the mesogloea.

Since the neurites of such a system are supposed to radiate in the thickness of the mesogloea, the plan is not essentially two-dimensional as the intra-epithelial net of the Hertwigs would be (Batham, Pantin, and Robson, 1960). The functionally separate muscle-sheets on each side of the mesogloea would thus not be controlled by functionally separate intra-epithelial nerve-nets, but would seem to depend for their independent contraction on some differential action within the common mesogloeal net. Although present physiological evidence does not support this idea, the presence or absence of mesogloeal nerve-cells is clearly fundamental to our interpretation of actinian behaviour in relation to the organization of the nervous system.

The question at issue is thus the presence or absence of a mesogloeal nerve-net in anemones. It is not an easy one to settle, for, if the Hertwigs are correct, it involves the proof of a negative: a difficult matter where the capricious staining of nerve-cells is involved, notwithstanding the much simpler histological picture which coelenterate epithelia present compared with the complex tissues of triploblastic animals. It is the primary object of this paper to consider the evidence about this problem.

The Hertwigs's work was based upon a very careful study of tissues treated with simple fixatives, such as strong alcohol and osmic oxide solutions and stained by such dyes as carmine derivatives (particularly alum and picro-carmine) and haematoxylin. Despite the simplicity of the methods, the reagents were exhaustively tested. They prepared the tissues for examination by making hand-sections and by the painstaking and ingenious use of maceration techniques. It is remarkable how much they discovered solely by means which today would be dismissed as inadequate.

Groselj (1909), whose work substantially supports the Hertwigs, used vital staining with methylene blue. Batham, Pantin, and Robson (1960) have since confirmed many results of the Hertwigs by their same methods. They also used vital staining with methylene blue, and made extensive use of a slight modification of Holmes's method (1942, 1943) by which the nerve-cells are stained with silver. This silver method was developed by one of us (E. J. B.) after unsuccessful tests with several other silver impregnation methods.

It appears significant that all those who have, on the other hand, claimed the presence of a mesogloeal nervous system in actinians have relied primarily upon silver impregnation methods. These are, unfortunately, liable to show bad fixation and precipitation artifacts, and for this reason we decided to make a fresh trial of several methods to see whether or not there was evidence of objects in the mesogloea which could be shown quite unequivocally to be nerve-cells.

Parker and Titus (1916), for example, used a drastic method in which the anemone was rinsed in distilled water and then passed directly to 5% silver nitrate for impregnation. To expose living marine tissues to distilled water and then to transfer them unfixed to silver nitrate might be expected to have disastrous effects on their histology. Our experience of this method bears out this expectation.

Havet (1901) used chiefly the rapid Golgi impregnation method by which the tissue is fixed by immersion in an osmic-bichromate mixture for several days and then impregnated in dilute silver nitrate. He notes the ease with which cells other than nerve and filamentous structures like nematocysts may take the stain.

Havet's work is extensive and his drawings are clear. Whatever the nature of the objects he stained, their existence is convincingly demonstrated. And many of the objects he figures in the epithelia are, as he points out, identifiable as the nervous elements seen by the Hertwigs. It is the structures in the mesogloea that are controversial.

Leghissa (1949, 1950) used a variety of methods which are not completely specified, but his figures of what he identifies as mesogloeal nerve-cells seem to be based mainly upon the Golgi–Kopsch silver method (Romeis, 1948, No. 1772); upon fixation with potassium dichromate and formaldehyde, followed ultimately by impregnation with 0·75% silver nitrate; upon direct impregnation with silver nitrate (concentration not specified) after fixation in de Castro's (chloral / alcohol / nitric acid) mixture; and on certain gold chloride preparations.

The work of Woollard and Harpman (1939) should also briefly be mentioned. After using a gold chloride / formic acid method on anemone and medusa tissues, and some methylene blue, these authors concluded that the coelenterate nervous system consisted of discontinuous cells and was not a fused network. But this support for our conclusions (Pantin, 1952; Batham, Pantin, and Robson, 1960) must be accepted with some reserve. In gold chloride / formic acid preparations, the formic acid distorts both muscle-fibres and mesogloea. Nor is it clear from their figures and their account that the structures studied by Woollard and Harpman were in fact nerve-cells.

Compared with all these, the modified Holmes's method used by Batham, Pantin, and Robson (1960) has a decided advantage in the quality of the initial fixation and in the absence of excessive precipitation of silver, so that much cytological detail can be seen compared with that found by more usual methods of fixation and staining.

In addition to these, certain other silver methods were used in the present investigation which, though they failed to reveal nerve-cells, yet gave good information about tissue histology. Willis's (1945) method, for example, is designed to cause a reaction between silver and histones and protamines in the nerve-cells. Tissue is fixed in ammoniacal formaldehyde in 70% alcohol and after paraffin sectioning passed again to formaldehyde, to acetic alcohol, and then to 0·1% silver, followed by hydroquinone and then gold toning and oxalate reduction. Peters's (1955*e*) method, involving fixation in formaldehyde / mercuric chloride followed by alkaline silver, sodium sulphite, and a glycine developer, was also tried.

It has been noted by one of us (Pantin, 1952) that when trying to identify nerve-cells in coelenterates we are faced with the difficulty that histological techniques giving good fixation rarely differentiate nerve-cells clearly from the surrounding tissues, while those which selectively stain nerves often produce artifacts and may fix tissues very badly. To be cogent, the circumstantial evidence in favour of the identification of nerve-cells must conform to the following criteria:

(1) The cells should stain by a variety of techniques in the same manner as nerve in other animals; and they should not stain in ways characteristic of other fibrous structures, such as collagen.
(2) Their cytological structure should be consistent with that of nerve-cells.
(3) Their anatomical relations should be consistent with the physiological evidence.

To this we may add:

(4) The structure should if possible be identified in the living state.

Applying these criteria Pantin (1952) and Batham, Pantin, and Robson (1960) have shown by several methods that there are well-defined networks of nervous and sensory neurites in actinian tissues. The term 'neurite' is used here to denote the process of a nerve-cell or sensory cell. It has been explained in a previous paper (Batham, Pantin, and Robson, 1960) that as the more usual terms 'axon' and 'nerve-fibre' are historically incorrect in this context, it is preferable to adopt the less specific word 'neurite' instead.

Unless such rules as these are applied there is a danger that other structures, both cellular and non-cellular, may be identified as nerve-cells. This is particularly the case with structures which, like neurites, are long and often fibrillar, such as muscle-fibres, the connective tissue-fibres in the mesogloea, nematocysts, and, as we shall show here, amoebocytes. As noted above, the danger is greatest in those very useful methods which are intended to deposit silver in the nerve-cell and its processes, since if fixation is poor there may be striking but false appearances due to precipitation of the metal on other fibrous structures.

There is the further problem that nerve-cells which can be identified with certainty may, nevertheless, be distorted on fixation so as to engender a misleading interpretation of their organization. Thus the neurites of the actinian nerve-net very easily become beaded during anaesthetization or fixation, or during vital staining. In this they resemble the axons of other animals. If they did not show such beading, the fact would cast doubt upon their being the processes of nerve-cells. Fig. 1, C shows a part of the retractor nerve-net of *Metridium* which has become beaded in this way. Similar beading can be seen to occur in time-lapse motion pictures of living chick neurites in tissue-culture (Hughes, 1953), and it is in fact reversible. Young (1944) showed that beading takes place in myelinated nerve when cytoplasmic viscosity and intracellular pressure are insufficient. The fine terminals of living non-myelinated nerve in the cornea are morphologically unstable (Weddell and Zander, 1951) and beading only becomes irreversible if it is caused by some adverse condition which has operated for too long (compare Bozler, 1927).

Beading is quite commonly seen in the neurites of *Metridium* after staining by Holmes's silver method. The fixation used in this method also tends to precipitate the axoplasm as neurofibrillar material, and lumps of such material within the beads can then simulate nuclei, as in fig. 1, C. Comparison of such appearances with other preparations leaves no doubt as to their true nature. Their close correspondence with certain similar structures figured by Havet (1901: compare, for example, fig. 1, A) in actinian nerve-cells indicates that the elements which he saw were also of this nature, and not chains of nerve-cells connected end to end as he suggests. Although such appearances may be particularly striking after silver impregnation, beading can occur at or before fixation.

Fig. 1, E shows a portion of nerve-net of the retractor face of a mesentery of *Metridium* stained by Holmes's silver method, in which beading is absent.

We shall now consider some of the artifacts that can be produced, first in the epithelio-muscular layer, and then in the mesogloea.

Mucus-cells. Fig. 2, D shows two mucus-cells in a mesentery stained with Batham–Holmes's silver method. These cells are common, and can be identified by comparison with preparations fixed in Susa and stained by polychrome staining methods, and by stains for mucin such as alcian blue. When fully developed they are goblet cells, and are anchored by a basal strand to the mesogloea. Without such identification, badly fixed examples might possibly be confused with ganglion cells. In well-extended mesenteries, for instance, they sometimes appear to have stellate processes, as in fig. 2, D.

Amoebocytes are also numerous in the epithelium, but will be considered later (p. 150).

Nematocysts. Actinian tissues are often pierced by the animal's own nematocysts, which discharge on fixation. Fig. 1, F shows nematocyst threads stained by methylene blue which have pierced the mesogloea of *Calliactis.* The even diameter of a discharged thread, its frequently wavy course through the tissues, and origin at a capsule are characteristic. Havet (1901) pointed out that, on fixation by the Golgi method, nematocysts may be shot through the tissues, and their threads may be impregnated with silver. Parker and Titus (1916) using their own silver method recognize this, but state that nerve-cells in the mesogloea can also be detected and can be distinguished from nematocyst threads. But they could detect no cell-bodies. Preparations made by us following their methods show structures which seem identical with those which they draw (fig. 1, D). Examination of our preparations leads us to conclude that in the great majority of cases these structures are without doubt nematocyst threads. The violent osmotic changes in Parker and Titus's method inevitably cause a widespread discharge of nematocysts. Possibly some of the structures seen may be due to deposition of silver on the collagen fibres of the mesogloea as well; we shall discuss this later (p. 151). We agree with Parker and Titus in finding no evidence of nerve-cell bodies by this method. Nor is there clear evidence of other cellular structure. Fixation is too poor, and the deposition of silver on fibrous remains is too great for any sound histological conclusions to be drawn from this method. The absence of clear detailed information is seen if we compare Parker and Titus's figures with those of the Hertwigs; or our fig. 1, D, repeating Parker and Titus's method, with our fig. 1, E by Holmes's silver method. The fibrous structures on which silver is deposited in the former do not appear to be nerve-cells, or even to be cellular, and in some cases the structures are unquestionably discharged nematocyst threads. It is therefore impossible to conclude that such preparations show nerve-cells in the mesogloea.

Muscle-cells. All silver methods we have tried are liable to stain the muscle-fibres (see fig. 3, A). These also stain vitally in due course with methylene blue, though they take much longer than do nerve-cells. As the preparation

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FIG. 1 (plate). A, one of Havet's illustrations (1901, pl. V, fig. 26) of a sectioned mesentery from *Metridium* prepared by the Golgi method.

B, illustration from Parker and Titus (1916, pl. I, figs. 5 and 6), showing horizontal sections of *Metridium* column.

C, beading neurites in the nerve-net of a *Metridium* mesentery, stained with Batham's modification of Holmes's silver method. Compare with Havet's figure above (A), and with E.

D, section of a *Metridium* mesentery stained with Parker and Titus's silver method. The dark lines are the shafts of nematocysts which have penetrated the mesogloea. Compare with Parker and Titus's illustrations above (B), and with F below.

E, part of a *Metridium* mesentery prepared by the Batham–Holmes silver method, in which the nerve-net shows little beading. Compare with C above.

F, section of *Calliactis* column, showing nematocyst threads discharged into the mesogloea. The tissues were stained for about an hour with methylene blue and fixed in ammonium molybdate, as for fig. 3, C. Compare with D above.

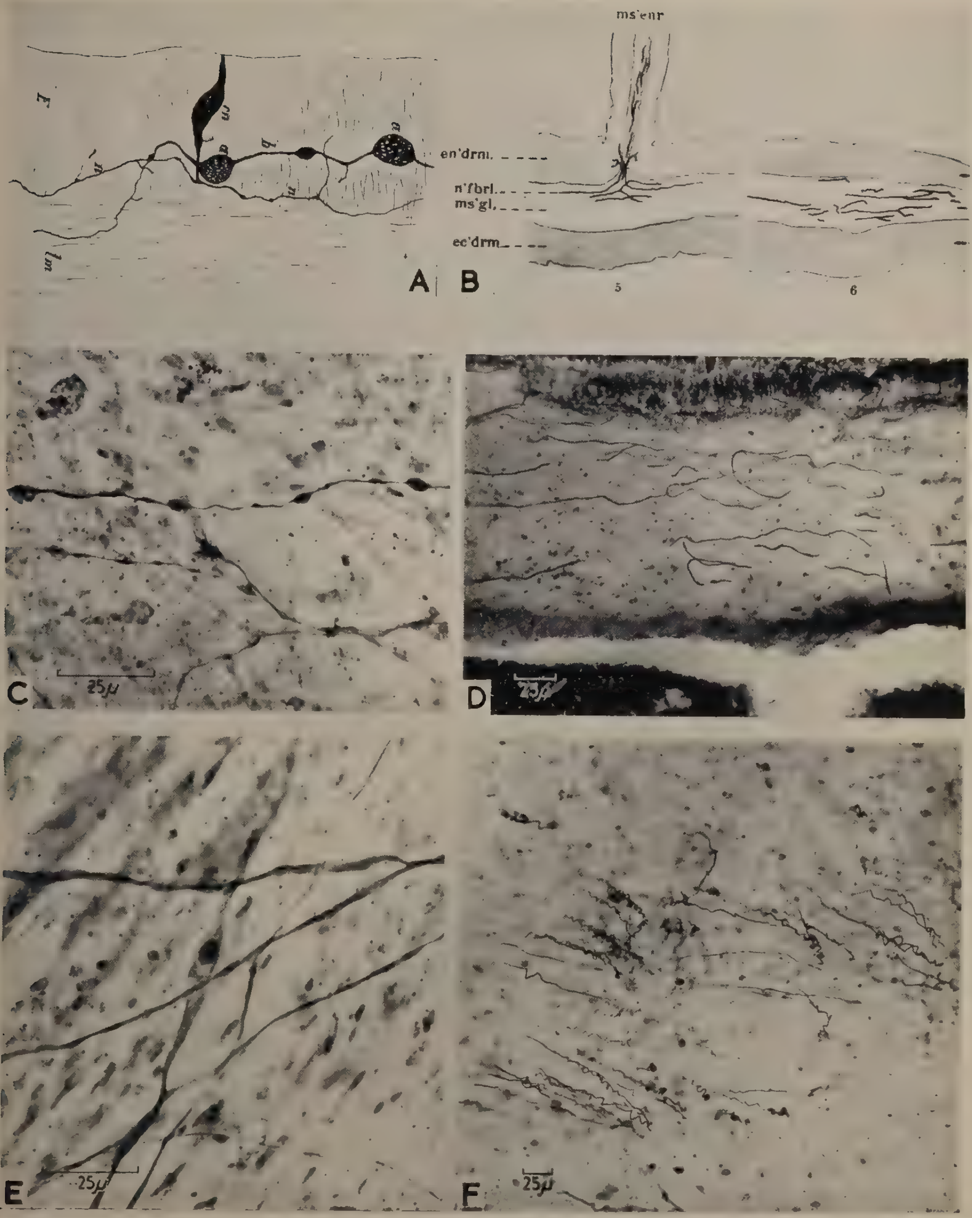

Fig. 1

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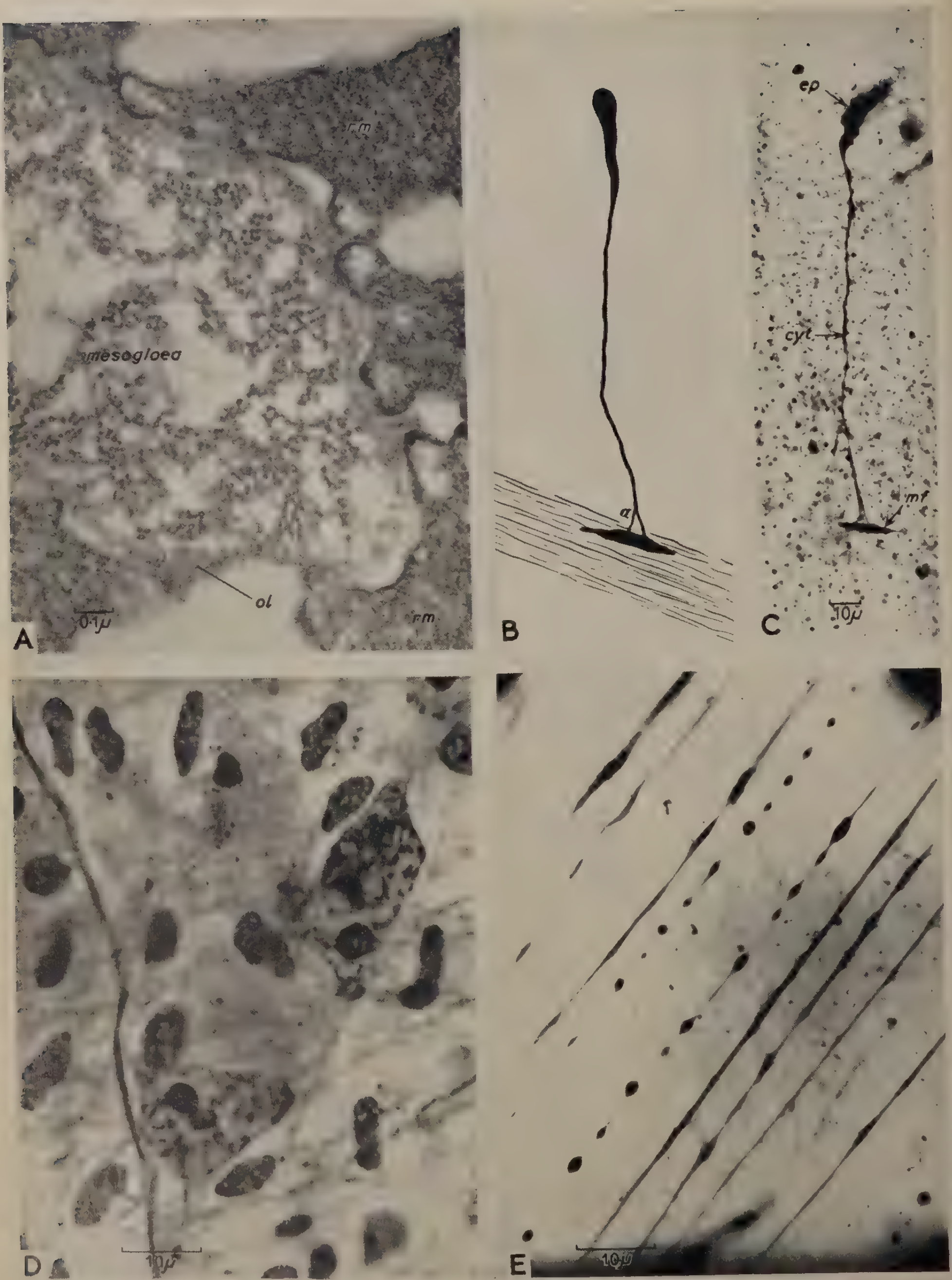

FIG. 2

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ages, beading occurs, with drop-like aggregations of cytoplasm along the muscle-fibre (fig. 2, E), though the character of the beading is not like that of neurites.

In all these features they show some resemblance to the histological behaviour of nerve-cells, but here again, accurate identification depends upon the avoidance of bad fixation and deformation. Provided this obtains, there is no difficulty in identifying muscle-fibres stained by these or any of the standard methods of polychrome staining. All give the same unmistakable picture of a muscle-sheet of numerous parallel fibres in close apposition to the surface of the mesogloea (Batham and Pantin, 1951; Grimstone, Horne, Pantin, and Robson, 1958). In agreement with the original observations of the Hertwigs (1879), it is important to realize how the layer of muscle-fibres is applied to the mesogloea at its surface. Fig. 2, A illustrates an electron micrograph of this for a portion of the retractor muscle-sheet of the mesentery. The same is true of the circular muscle-layer of the column (Batham and Pantin, 1951). Figures of this in some textbooks are misleading.

This point can be important particularly because with some methods, such as that of Golgi impregnation, sometimes only individual muscle-fibres will take up the stain, and it may be a matter of great difficulty to determine the relations of the muscle-cells to any mesogloea over which they lie. This is illustrated in fig. 2, C, a Golgi preparation from the sphincter region of *Metridium*. As the Hertwigs showed, the endodermal muscle-cells are all characteristically epithelio-muscular cells, the muscle-fibre being connected to the epithelial part of the cell by a cytoplasmic neck. In fig. 2, C this epithelial portion and part of the muscle-fibre of isolated muscle-cells have stained with silver and it is not easy to tell at just what level in the tissue they are. Havet (1901) illustrates just such structures by the same method, as shown in fig. 2, B. He, too, identifies them as epithelio-muscular cells, but figures the muscle-fibre as though it were embedded in the mesogloea. Careful examination of such cases convinces us that this is an optical misrepresentation due to isolated densely stained muscle-fibres of the muscle-sheet lying above the mesogloea and not within it. Like those of the Hertwigs, our own visual observations are entirely consistent with the muscle-fibre / mesogloea relation disclosed in fig. 2, A.

FIG. 2 (plate) A, electron micrograph of part of a transverse section of a *Metridium* mesentery. Retractor muscle-fibres (*rm*) are closely applied to the underlying mesogloea, within which the sectioned mesogloeal fibres form a distinct outer layer (*ol*).

B, one of Havet's figures of an epithelio-muscular cell from a *Metridium* mesentery (pl. V, fig. 34), from material sectioned after staining by the rapid Golgi method.

C, epithelio-muscular cell from the sphincter region of a small *Metridium* sectioned after staining by the rapid Golgi method. The epithelial part of the cell (*ep*) is connected by a cytoplasmic process (*cyt*) to the muscle-fibre (*mf*), only a short part of which occurs in this section.

D, two mucus-cells in a mesentery of *Metridium* stained with Batham's modification of Holmes's silver method. A neurite (left) and epithelial nuclei are also seen.

E, longitudinal muscle-fibres from a whole mesentery of *Metridium* showing beading due to excessive stretching of the preparation. Compare fig. 1, C. Susa, iron haematoxylin.

Amoebocytes. Among cells which will take up methylene blue in actinians are amoebocytes, which are wandering cells found throughout the mesogloea, ectoderm, and endoderm. Their appearance is extremely variable but it seems to depend on external conditions, so that their form is consistent within any preparation. Thus in one preparation all the amoebocytes may look more or less rounded. In others they may appear elongated with fine pseudopodial extensions, often running in some common direction related to the fibrous structure of the mesogloea. Although their functions are imperfectly known, it may be inferred that the amoebocytes are part of only one physiological system.

The amoebocytes often become elongated, especially in deteriorating tissues, and their long protoplasmic filaments may extend through the mesogloea or epithelium. The cells may stain with silver or methylene blue and thus show a confusing resemblance to nerve-cells. In this guise they have misled several authors who believed that nerve-fibres penetrated the mesogloea. Sanchez (1918), however, pointed out the similarity between the mesogloeal 'connective tissue cells' in his preparations of *Sagartia* (= *Calliactis*) *parasitica* and the mesogloeal 'nerve-cells' of *Metridium* described by Havet (1901). Havet's drawings are so clear that his so-called 'nerve-cells' in the mesogloea can be identified as amoebocytes by their thin, branching processes alone. If the figures denominated coelenterate nerve-cells by Leghissa (1949, 1950) and Torelli (1952) are compared with what are demonstrably amoebocytes in fairly well-fixed tissue (fig. 3, B, D), the correspondence in form and situation is at once evident.

Amoebocytes stained with methylene blue can be distinguished from nerve-cells by their smaller size, and by the fact that their processes branch and may apparently end on mesogloeal fibres, and are more tenuous than axons. The large nucleolus of the nerve-cell is absent, the nucleus stains far less readily, and in well-fixed preparations the cytoplasm is consistently granular. The granules are strongly fuchsinophil (Chapman, 1953); their presence at once identifies amoebocytes in electron microscope sections (Grimstone and others, 1958). When stained with silver by Holmes's method, their cytology is manifestly different from that of nerve-cells. In living tissues amoebocytes may aggregate locally, and individual cells mounted on a slide in sea-water have been observed to migrate. Unlike nerve-cells they are therefore mobile.

As mentioned above, these mesogloeal cells often assume a spindle-shape,

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FIG. 3 (plate). A, transverse section of *Calliactis* column stained by Willis's silver method. Mesogloeal fibres and amoebocytes have stained, as well as muscle-fibres (upper left).

B, whole mount of *Metridium* mesentery, showing amoebocytes with irregular processes. Compare to A and D. Batham–Holmes silver method.

C, section showing beaded fibres in the mesogloea of the sphincter region in *Calliactis*. Slices of living tissue were treated with menthol overnight, stained with methylene blue for about an hour, fixed in ammonium molybdate, and subsequently sectioned.

D, amoebocytes in a whole mesentery of *Metridium*. In this preparation they show few stellate processes. Epithelial nuclei and muscle-fibres (right) are also visible. Baker's formaldehyde-calcium / chlorazol black E.

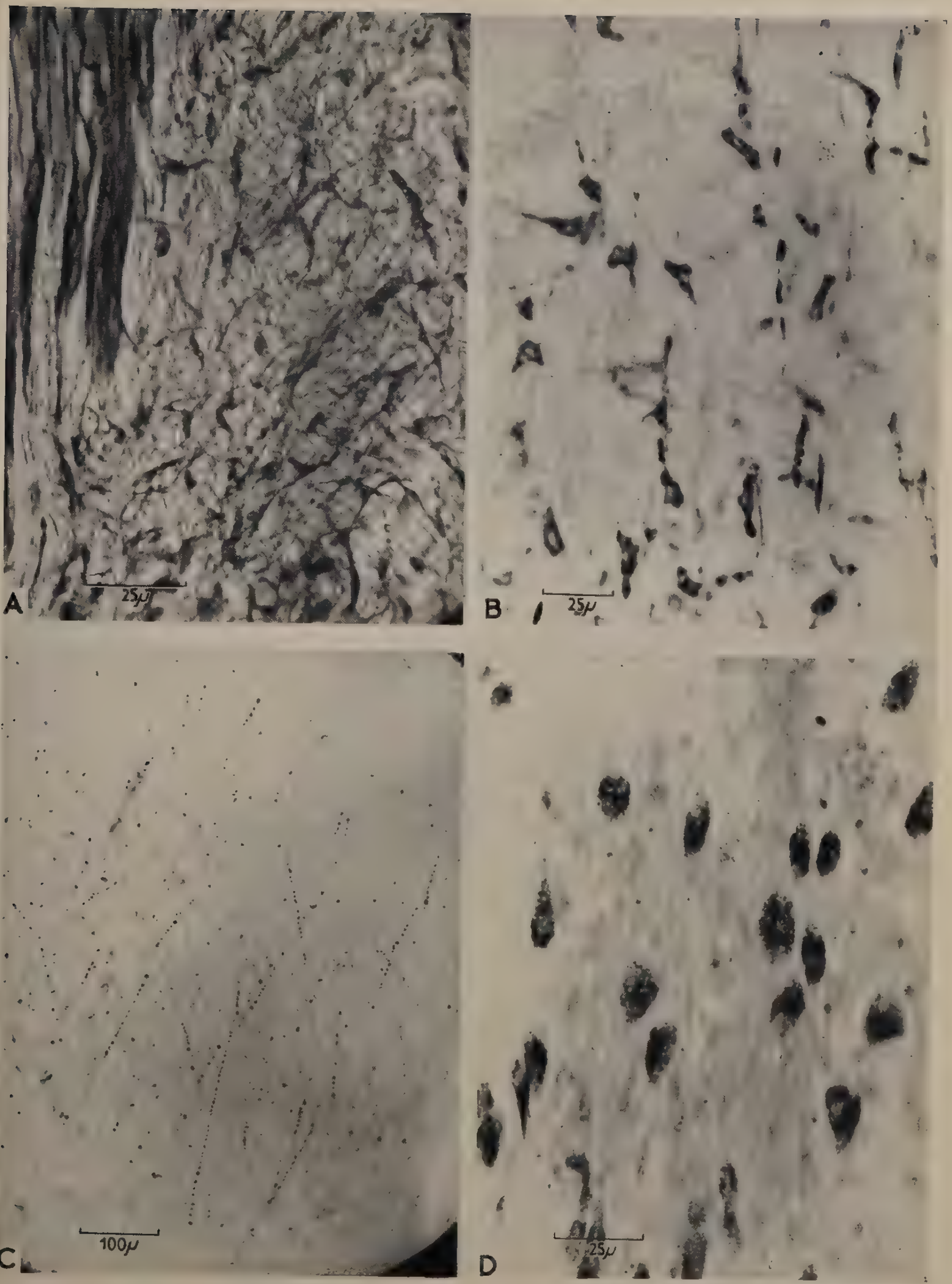

FIG. 3

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and may then resemble small bipolar nerve-cells. Their cytoplasm may be drawn out into long processes which carry droplets at intervals. On inspection, however, these processes are seen to be very much finer than most nerve axons, and they may branch. They often anastomose with each other, and may end, apparently, on a mesogloeal fibre. Nothing resembling a synaptic junction can be seen. These features are all foreign to nerve-cells. On the other hand, living mesogloeal cells will sometimes stain with methylene blue. Chapman (1953) records the same for *Calliactis* and *Aurelia*. But the staining time is usually much longer than that of recognizable nerve-cells in the same preparation, and in silver preparations mesogloeal cells are also quite distinct from nerve-cells. It may therefore be concluded that cells found in the mesogloea show none of the morphological characteristics peculiar to nerve-cells, and certainly cannot function as such.

Mesogloeal fibres. It has been shown by Chapman (1953) that the mesogloea of anemones and medusae is an organized structure of collagen-like fibres. In anemones they are arranged in a crossed-lattice pattern, and, in the column at least, this appears to be a mechanical result of the animal's movements. The physical properties which they share with fibres of any kind sometimes make them stain deeply or receive precipitated silver with methods intended for nerve-cells (compare Peters, 1955*a*; Zon, 1936). Many fibres seem to provoke the deposition of silver and may therefore stain.

With some silver methods, the combination of the staining of the mesogloeal lattice fibres and of elongated amoebocytes associated with it can give pictures suggestive of a complex organized three-dimensional nerve-network (fig. 3, A). The collagen lattice follows a well-defined pattern (Chapman, 1953), and amoebocytes are often found following its strands. But if fixation and cytological preservation are good the deception is easily detectable even in a single preparation. Nor can it survive the results of comparing preparations stained in different ways.

A comparison of Leghissa's figures (1949) with preparations such as that of fig. 3, A strongly suggests that Leghissa's mesogloeal nerve-cells and their neurites are in fact a similar artifact network derived from the deposition of silver on the collagen lattice and embedded amoebocytes in which the preservation is not sufficiently critical to distinguish this. It is noteworthy that his figures do not show any cells as unequivocally nervous in character as in the Hertwigs's illustrations or the photographs of Batham, Pantin, and Robson (1960).

Other mesogloeal structures. The only other structures which suggested to us the possibility of mesogloeal nerve-fibres were certain long-beaded structures occasionally seen traversing the mesogloea. They sometimes appear when hand-sections of *Calliactis* are stained vitally with methylene blue for several hours. Fig. 3, C shows such structures from the sphincter region.

A careful study of these fibres has never revealed a cell-body in them. Moreover, we were able to show that they do not contain normal cytoplasmic staining elements. We photographed 20-μ sections of this sort of vitally stained preparation fixed with ammonium molybdate, and identified landmarks in each section. We then re-stained them with Mallory's triple stain or Masson, and rephotographed the same area. We were able to identify parts of the fibre, but the structures stained identically with the surrounding collagen and appeared to contain no cytoplasm.

A careful examination of silver sections sometimes shows beaded mesogloeal structures which might possibly correspond to these methylene blue fibres.

The Validity of Silver Methods

It is now appropriate to discuss certain aspects of the four criteria by which nerve-cells may be identified (p. 146).

The first, that they should stain like the nerve-cells of other animals after a variety of methods, is the most difficult to establish, and this is not only because special methods for nerve-cells tend to be capricious and may stain other structures of the kinds which have been discussed, but also because the reasons for which special methods may be successful are not yet fully understood. Recent studies of the use of methylene blue as a vital stain, and of the mechanism of silver staining, have contributed several improvements in technique; but it is not yet clear how the properties of nerve-cells are responsible for their differential staining.

In a comprehensive review of peripheral nerve-terminations in mammalian tissues Weddell, Palmer, and Pallie (1955) have considered the problem of artifacts in detail. These authors analyse the extensive literature, and are able to infer much about peripheral nerve that is also demonstrated by their own work. They find that both methylene blue and silver staining are enhanced if the tissues are treated with hyaluronidase, which aids penetration of the dyes or fixative, and greatly reduces artifacts (Weddell and Pallie, 1954). Unstained nerve-cells studied under phase contrast provide a standard for reference. Although different techniques have been used to study the actinian nerve-net, and a trial of hyaluronidase did not improve staining with this material, our conclusions and those of Weddell and others as to the origin of certain artifacts are much the same.

It is well known that the numerous silver methods available to histologists are usually employed according to the type of structure that is to be stained. Some are more appropriate for the central nervous system, others for peripheral nerve (Carleton and Drury, 1957), and others are used for connective tissue. Methods for the vertebrate central nervous system often aim at the selective staining of relatively few elements in a large mass of identified nervous tissue. But when peripheral nerve is concerned, the aim is to stain the system as completely as possible. The nerve-cells are relatively few in number, and are usually scattered among other tissues. It is not yet understood why some empirical silver techniques can stain different types of nerve-cell, apparently according to their function. It is worth noting, however, that the silver methods used by Havet, by Parker and Titus, and by Leghissa for actinians are more usually employed for the vertebrate central nervous system, whereas the silver method of Holmes (1942, 1943), which has been modified to demonstrate the nerve-net of *Metridium*, is especially suited to the staining of peripheral nerve.

The recent study by Peters (1955) of the mechanism of silver staining is based on sections of vertebrate central nervous material. His method, while successful with these tissues, did not stain any nerve-cells in preliminary trials with actinian material. Peters's experiments show that specificity of staining depends particularly on the developer used, and also on the pH, temperature, and concentration of the silver impregnating solution. Reducible silver, which as suggested by other workers combines mainly with histidine, is by no means confined to nerve-cells. Peters accepts Zon's earlier conclusion (1936) that tissue components exert a protective action on silver staining. This means that certain unspecified protein elements in the section somehow retard the reduction of silver at particular sites, but this interesting suggestion is not discussed further.

The problems of selectively staining either a few particular nerve-cells or all of those present are difficult. Methods which tend to stain all the nerve-cells present may stain other structures as well. In this respect, the work of Irving and Tomlin (1954) on the argyrophil properties of collagen and reticulum is of special interest. These authors used only the silver methods of Foot and Gomori, and experimented with connective tissue preparations before staining them. They showed that the staining of connective tissue-fibres depends as much on the chemical properties of the ground substance as on those of the fibres. Thus treatment with various proteins and with hyaluronic acid intensifies staining. The silver particles are seen by electron microscopy to be deposited only on the surface of the fibres, whereas in the case of nerve-cells, Peters (1955*d*) found that silver is reduced within the axon as well as on the surface.

All this bears on two more of the criteria for the identification of nerve-cells (p. 146), their cytological structure and anatomical relations. Neither of these can be adequately studied without specific staining methods, or without taking precautions to minimize artifacts. Histochemical methods and electron microscopy should be able to provide new information about the actinian nerve-net, and should help to clarify the function and distribution of amoebocytes, with which nerve-cells may still be confused. This confusion does not seem to be completely resolved in a recent paper of Leghissa and Mazzi (1959). In this, cells that we have broadly referred to as amoebocytes until there is some evidence about their function (Batham, Pantin, and Robson, 1960), are subdivided into mesocytes and amoebocytes. Although seven points of comparison by which nerve-cells differ from mesocytes are tabulated, and although a variety of optical and staining methods has been used to study the mesogloea, the physiological properties of nerve-cells are not mentioned, and their identification by this author is correspondingly difficult to confirm. It is hoped that future work which is mentioned in this publication will throw further light on the question of mesogloeal nerve-cells. The fact that both unstained nerve-cells and amoebocytes can be distinguished in living mesenteries of *Metridium* is relevant to this, and is one of the decisive criteria for their identification (p. 146).

THE PRESENCE OR ABSENCE OF MESOGLOEAL NERVE-CELLS

It is hard to prove a negative. But the results of many years' work by us seem unequivocally to support the position of the Hertwigs (1879) that there are no mesogloeal nerve-fibres in the actinian tissues we have investigated. The observations of Groselj are also unambiguous. Whether that is true of other cnidarians is another matter. As the Hertwigs pointed out, the actinian sensory-nervous system is organized more simply than in medusae (compare Pantin, 1956). In Hydromedusae, for example, the two subumbrellar nerve-rings are connected by neurites which cross the mesogloea (Hertwigs, 1878). Mackie (1960) has reported the presence of mesogloeal nerve-cells in *Velella*, at least between the epithelia of the outer surface and invaginated float ectoderm.

Our own investigations (Batham, Pantin, and Robson, 1960), using methylene blue, silver, and other methods, have so far shown that at least in the mesenteries and column of *Metridium*, the sensory-nervous system is intra-epithelial. This is particularly clear in silver preparations in which the epithelium with its stained nerve-net is stripped off the underlying mesogloea, which can then be seen to possess no stained nervous elements. It should be emphasized, however, that the structure of the nerve-net in the disk and sphincter, and in the foot, has not yet been examined. It is hoped to extend the histological study of the mesenteries and column to these regions.

Present histological information is in full agreement with the physiological evidence. As noted earlier (p. 144), the ectodermal and endodermal muscle-sheets of the oral disk are functionally separate, and so are the radial and retractor muscle-systems of the mesenteries. In the column, the physiological independence of the endodermal sensory-nervous system from ectodermal elements has been conclusively demonstrated by removal of the ectoderm in living *Calliactis* (Passano and Pantin, 1955).

The clearest account of mesogloeal nerve-cells in actinians is that of Havet (1901). The epithelial elements he describes correspond in a great measure with those of the Hertwigs, Groselj, and ourselves. When structures in the mesogloea which he identifies as nerve-cells are compared with our own preparations according to the rapid Golgi method, there is little doubt that amoebocytes and the fibrous network of the mesogloea can take up silver to produce these images. The same is true of the figures of Leghissa (1949). The method of Parker and Titus (1916) we have found histologically wholly unsatisfactory. None of the figures illustrating their work shows any nervous elements such as the Hertwigs and we have found: the fibres in the mesogloea that Parker and Titus call 'neurofibrils' closely resemble nematocyst threads and mesogloeal fibres. This conclusion is important because their work is cited in textbooks as evidence of mesogloeal nerve-cells in actinians (Hyman, 1940).

Our conclusion thus wholly supports the intra-epithelial character of the nerve-net in the actinian material which we have studied. We also confirm the essential separateness of the epithelia from the underlying mesogloea, and the general absence of cells in that tissue other than amoebocytes. The only other cells which from time to time are truly to be found within the mesogloea are the genital cells (Pantin, 1960).

Much of this work was done at the Marine Biological Laboratory, Plymouth, to the Director and staff of which we are most grateful for the many facilities given us. We wish to thank the Department of Scientific aud Industrial Research for grants for the development of a special research which enabled this work to be carried out, and the Council of Trinity College, Cambridge, for a contribution to the cost of the plates.

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A Comparative Histological Study of the Tube-feet of two Regular Echinoids

By DAVID NICHOLS

(*From the Department of Zoology, University Museum, Oxford*)

With one plate (fig. 8)

SUMMARY

The histology of the suckered and peristomial tube-feet of the two regular echinoids *Cidaris cidaris* (Cidaroida) and *Echinus esculentus* (Diadematoida) is described; of these orders the Cidaroida is the more primitive. The suckered tube-feet of both urchins have a connective-tissue sheath the fibres of which branch extensively before inserting at the disk, and in which are embedded numerous spicules, enlarged distally to form a supporting skeleton of the disk. A series of levator muscles, separate from the retractors of the stem, raise the centre of the disk during adhesion, and this activity also probably squeezes mucus from a series of glands opening at the disk surface. In *Cidaris* a second series of glands, goblet cells in the disk epithelium, are operated by special short muscle-fibres running between them; these cells and the muscles are absent in *Echinus*. In *Cidaris* sensory cells are apparently scattered over the entire disk surface, whereas in *Echinus* they appear to be mainly concentrated in a ring round the disk periphery. The peristomial tube-feet of both urchins are not suckered; the levator muscles are absent, and the disk, supported by a less complex calcareous skeleton, contains mainly sensory cells and mucous glands. The possible derivation of the diadematoid, clypeasteroid, and spatangoid tube-foot plans from that of the cidaroid is discussed. The differences in ornamentation of the regions of the test which bear the tube-feet are discussed functionally, the main conclusion being that a tube-foot whose activity is at all angles to the test requires a wider base than one whose activity is mainly perpendicular; this is shown to be the case in spatangoids also. A respiratory function for Stewart's organs in the Cidaroida is suggested.

INTRODUCTION

THIS paper is a further part of a comparative histological study of the tube-foot / ampulla systems in echinoderms. With the exception of some aspects of their histology and function in certain asteroids, these remarkable and unique hydrostatic organs have received comparatively little attention in the past, and no attempt has been made to homologize structures in an evolutionary sequence, as will be done briefly here. It will be shown in this paper that the tube-feet of a primitive echinoid have a histological structure from which can be derived that of an advanced regular echinoid and the various types of tube-feet in a clypeasteroid and a spatangoid.

As far as one can tell from hard parts alone, the genus *Cidaris*, of the order Cidaroida, is quite close to the fossil genus *Miocidaris*, which, as Jackson (1912) shows, is the only echinoid genus found on both sides of the geological crisis marking the junction of the Permian and Triassic systems. This being

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so, all subsequent echinoids, regular and irregular, presumably have a cidaroid ancestry, and it is very fortunate that cidaroid material has become available during the course of this work. The species whose tube-foot structure is described here is *Cidaris cidaris* (Linn.) (fig. 1), which occurs in the north-eastern Atlantic and the Mediterranean. As representative of another regular echinoid order with living members, the Diadematoida, the common British sea-urchin *Echinus esculentus* Linn. is chosen.

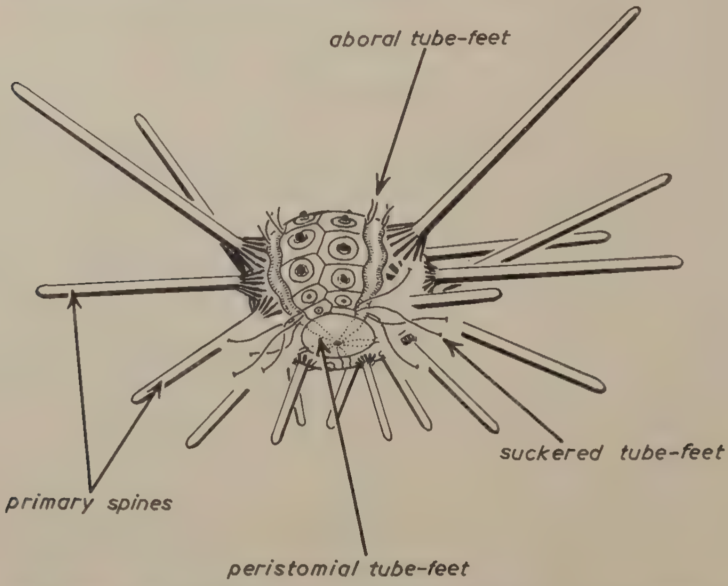

Fig. 1. Diagrammatic drawing of *Cidaris cidaris*, with the spines of two ambulacra and one interambulacrum removed for clarity. A few only of each series of tube-feet are drawn: the rest are indicated by their pores.

Material and Methods

The specimens of *C. cidaris* were dredged from the edge of the continental shelf in north Biscay, 51° 15′ N., 11° 40′ W., at a depth of about 500 fathoms, and were placed in sea-water tanks at the Plymouth laboratory, where they lived perfectly well for several years. *E. esculentus* was dredged from the shell-gravel of the Looe–Eddystone ground.

The tube-feet were removed from the urchins by cutting as close to the test as possible. The fixative used was Heidenhain's 'Susa', made up in sea-water, the tissues being fixed for 6 to 8 h. Susa gave good cytological results and was also adequate for decalcification. Before fixation, some of the animals were narcotized in propylene phenoxytol (Owen, 1955), by the same technique as that used for *Antedon* (Nichols, 1960). Embedding and staining followed the same procedure as that used for the study of the tube-feet of irregular echinoids (Nichols, 1959 *a*, *b*). For ordinary histological detail Pantin's (1948) modification of Masson's trichrome technique was used.

For a study of the calcareous plates and spicules 6 tube-feet of each type were treated on a slide with 2% hot caustic potash.

THE ARRANGEMENT AND STRUCTURE OF THE PLATES BEARING THE TUBE-FEET

In all present-day echinoids there are two columns of plates in each ambulacrum and two in each interambulacrum. The cidaroid ambulacra are simple, that is, each plate bears a single pair of pores in uniserial arrangement (fig. 2, A).

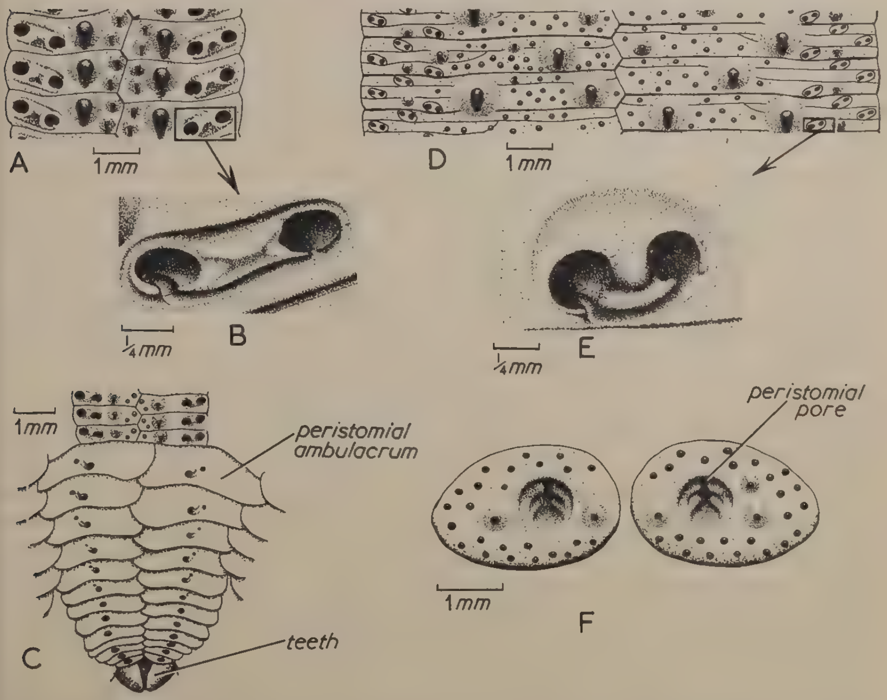

FIG. 2. Drawings to show the arrangement and structure of the pores from which the tube-feet arise in *C. cidaris* (A, B, and C) and *E. esculentus* (D, E, and F). A, part of the adoral region of one ambulacrum of the denuded test of *C. cidaris*, showing the monoserial arrangement of the pore-pairs. B, enlarged view of one pore-pair from A, as indicated. C, part of the peristome, showing the arrangement of the pores in the peristomial region of one ambulacrum. D, part of the adoral region of one ambulacrum of the denuded test of *E. esculentus*, showing the triserial arrangement of the pore-pairs. E, enlarged view of one pore-pair from D, as indicated. F, one pair of plates from an ambulacral region of the peristome of *E. esculentus*, showing the pores which bear a pair of peristomial tube-feet.

Each pore-pair (fig. 2, B) is surrounded by a furrow and the individual pores have a slight pyramidal swelling between them; this ornamentation is chiefly for the attachment of muscles of the tube-foot stem. The furrow on the oral side of each pore-pair is slightly deeper than elsewhere, and turns abruptly towards the interior of the test through a notch on the oral side of the perradial pore (that nearest the mid-line of the ambulacrum). A similar, though very much smaller notch occurs in the oral side of the other pore. Both ambulacral and interambulacral plates are continued across the peristomial edge on to the peristome itself (fig. 2, C), though here they do not abut against each other to form a rigid structure but imbricate in such a way that the peristome is flexible. The two columns of plates in each interambulacrum are usually, but not always, fused into one column in *C. cidaris* (not shown in fig. 2, C). Whereas the pore-pairs of the test are placed so that a line joining each pore is roughly meridional, those of the peristome gradually transform adorally until a line joining them is parallel to the long axis of the ambulacrum; also, the perradial pore becomes larger, while the other becomes very much smaller than the coronal pores and may even disappear entirely at the mouth edge.

In the diadematoids the ambulacra are compound, that is, each plate really consists of two or more plates 'crushed' (Hawkins, 1919) together (fig. 2, D). In *E. esculentus* the pore-pairs are in triserial arrangement. There is a furrow surrounding each pore-pair, as in cidaroids, but in this group it is shallower and very much wider on the aboral side of the pores (fig. 2, E). On the oral side, as in *Cidaris*, the furrow leads into the perradial pore and thence to the interior of the test. Immediately aboral to this furrow is a second one parallel to the first which probably corresponds with the oral side of the pyramidal projection in *Cidaris*. In the diadematoids the peristomial plates are not visible on the surface of the living animal, as they are in cidaroids: they are very much reduced and are embedded in thick connective tissue. One pair of plates in each ambulacrum, however, is larger than the rest; these lie close to the rim of the mouth and each is pierced by a single pore bearing a tube-foot. Each pore (fig. 2, F) has a cavity which is nearly subdivided into three by ridges projecting from two opposite sides. The actual hole through the plate enters it from the inside near its aboral edge; it pierces it at an angle because the branch from the radial water-vascular canal to each peristomial tube-foot lies against the inside surface of the peristome and has to turn through nearly 90° as it passes through the plate to become the lumen of the peristomial tube-foot.

THE ARRANGEMENT AND MORPHOLOGY OF THE TUBE-FEET

Though there is not nearly so much division of labour in the tube-feet in any one ambulacrum of regular echinoids as in the irregulars, there is a division into two distinct types: first, the principal tube-feet which arise from the pore-pairs of the test, and, secondly, the peristomial tube-feet. The principal tube-feet normally have suckered disks, though the suckers may become weak and even absent altogether towards the aboral pole, particularly in cidaroids. Those with suckers are principally organs of locomotion, anchorage, and respiration, with the subsidiary function of sensation, while aborally the functions of locomotion and anchorage become less and less important. The tube-feet of *C. cidaris* are far less important in locomotion than are those of *E. esculentus*: the spines move the animal while the tube-feet act as movable 'guy-ropes'; but it is hard to see why the cidaroid sucker is less developed, because even a guy-rope requires a strong tent-peg. It is a feature of the cidaroid suckered tube-feet that while they are extending free in the water the centre of the disk (the diaphragm) is raised as a conical projection (see, for instance, Prouho, 1887, plate XIV, fig. 5). This may simply be caused by pressure of water in the lumen against the diaphragm, and the reason why it is never seen in diadematoids would be because the diaphragm is much stronger.

Though they have no suckers on their disks, the peristomial tube-feet resemble at a glance those of the test in external morphology; indeed, Prouho (1887) incorrectly states of *C. cidaris* that the series of suckered tube-feet continues orally on to the peristome, and that 'on sait qu'il n'existe chez les Cidaridés rien de comparable aux dix tentacules buccaux des Échinidés', from which it is clear that though he recognized the two sorts of tube-feet of diadematoids ('Échinidés') he failed to realize that all the peristomial tube-feet of cidaroids are without suckers. Mortensen (1928), on the other hand, regards only the innermost of the peristomials of cidaroids as corresponding to what he calls 'buccal' tube-feet of other regulars. It is true, as Mortensen himself noticed, that the 10 innermost of this series are slightly bigger than the others, but there is no doubt, from the present work, that morphologically and histologically all the peristomial tube-feet of *Cidaris* are similar. Their functions are most probably sensation and respiration, as will be discussed later.

It is relevant to clarify here the terminology of the tube-feet near the mouth. The term 'peristomial tube-feet' refers only to those which pierce the peristomial membrane; in clypeasteroids (see, for instance, Nichols, 1959*b*) the sensory tube-feet round the mouth arise directly from the circum-oral water-vascular ring, so it is uncertain whether they are homologous with the peristomials of regulars; for this reason they are referred to as 'buccal tube-feet' in this work. Finally, the organs of feeding in each ambulacrum of spatangoids (see, for instance, Nichols, 1959*a*) arise from the radial water vessels; though they may, for this reason, be homologous with the peristomials of regulars, this is by no means certain, since the peristomials have no ampullae; in any case, their morphology and function is so different that they are referred to as 'feeding tube-feet'.

THE HISTOLOGY OF THE TUBE-FEET OF *CIDARIS*

The suckered tube-feet

The stem. The wall of the stem (fig. 3) consists of 4 layers: an outer epithelium, a connective-tissue sheath, a muscle-layer, and a coelomic epithelium. The main framework of any tube-foot is the connective-tissue sheath, and this is a convenient starting-point in a description of the anatomy. The function of the sheath, as Smith (1947 *a*, *b*) showed for asteroids, is to allow the tube-foot to extend considerably while still keeping the lumen diameter constant. The sheath in this case has 3 components. The outermost layer is the most diffuse, with mainly longitudinally running fibres; in it are normally embedded many

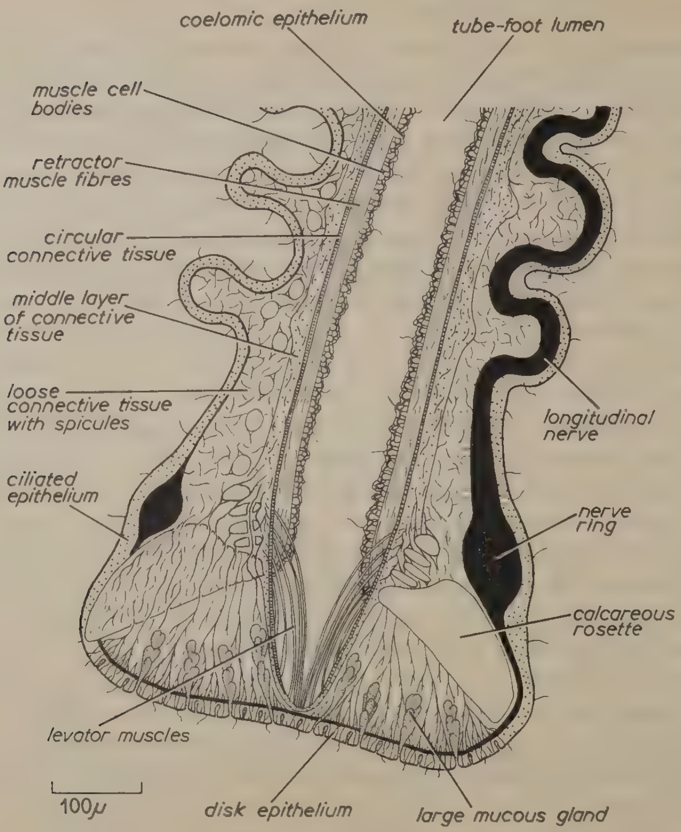

FIG. 3. Diagrammatic longitudinal section of the distal end of a suckered tube-foot of *C. cidaris*. The section has been taken through the longitudinal tube-foot nerve (shown on the right of the stem). On the right side of the disk the section has been taken through one of the indentations in the edge of the skeletal rosette in which a branch of the disk nerve-plexus lies, while on the left the section shows the passage of connective-tissue strands through the skeletal frame and rosette.

coelomocytes which have probably migrated there from the water-vascular canal, and a large number of curved spicules. The latter are arranged roughly in two longitudinal columns (fig. 4) which overlap on one side but between which there is a gap on the other: the longitudinal tube-foot nerve runs in this space. The spicules themselves have thorny processes radially projecting from them, and these processes become particularly prominent towards the tube-foot disk. At the distal end of the stem the spicules become closely-packed and very much flatter. This happens at just about the level of the nerve-ring, that is, just distal to the end of the longitudinal nerve, and here the spicules form a complete ring. The arrangement is not haphazard; there are 5 interlocking sets of spicules in the ring, each set being placed across the junction between adjacent plates of the rosette of the disk, which will be described later. There is no sharp dividing line between the outermost connective-tissue layer and the next. The middle layer of the sheath is fairly dense and consists of fibres arranged mainly longitudinally, though they curve round each other at intervals to form an interwoven net and also send fibres into the outermost layer. Nearer the disk they become much more regularly longitudinal in arrangement, and, together with fibres of the outer layer, pass through the skeletal rosette of the disk (see later) to fan out before inserting at the disk epithelium. At the centre of the disk (the diaphragm) some fibres of this layer form a cap round the distal end of the tube-foot lumen. The innermost layer of the three is the thinnest and most dense, and consists entirely of circularly running fibres. It is quite distinct from the middle layer.

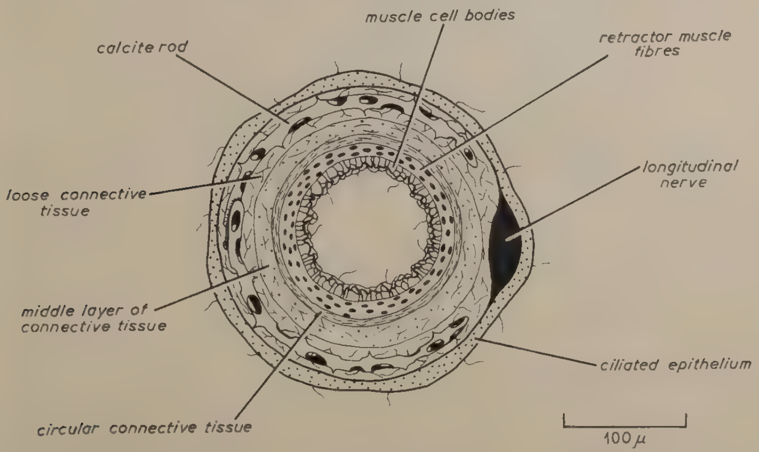

FIG. 4. T.S. of the retracted stem of a suckered tube-foot of *C. cidaris*, showing the arrangement of the two columns of spicules in the loose outer connective-tissue layer in relation to the longitudinal tube-foot nerve.

It appears that in this tube-foot there is no space between any of the component layers to allow for extension, and comparison of narcotized and non-narcotized material suggests that the connective-tissue layer as a whole can crumple and distort sufficiently to accommodate the folding of the epithelium while the muscles contract. The thickness of any of the connective-tissue layers in a section, therefore, will depend on the state of distortion, but it seems as though the whole layer in the contracted state occupies between 100 and 200 μ, and in the expanded state between 10 and 20μ.

Outside the connective-tissue sheath is the external epithelium and its underlying nerve-plexus. Unlike any other tube-foot so far studied in this work, this epithelium is quite heavily ciliated, the 10 to 15 μ long cilia being spaced every 3 to 5 μ or so. The cells of the epithelium are heavily vacuolated and interspersed with coelomocytes, giving the epithelium a diffuse appearance. The sub-epithelial nerve-plexus is so thin as to be virtually invisible, except where it widens out to form the longitudinal nerve (fig. 4). This nerve is supported by neuroglia, running mainly radially from the diffuse connective-tissue layer to the basement membrane of the epithelium. At the distal end of the tube-foot stem the nerve-plexus widens out to form the nerve-ring; this too is strengthened by neuroglia.

Next internally to the connective-tissue sheath is the longitudinal retractor muscle. In the contracted state this is normally about 50 to 80 μ thick, and the individual fibres between 2 and 6 μ thick. The fibres originate and insert at the connective-tissue sheath at intervals down the length of a tube-foot, so that in the contracted state the fibres loop in towards the lumen. As usual in tube-foot muscle (Nichols, 1959*a*) the fibres are surrounded by an exceedingly thin layer of cytoplasm down most of their length, but in one place there is a bulge, the cell-body, containing the nucleus. The interesting feature about the muscle-layer in this case is the position of the cell-bodies and nuclei. Here, they are interspersed among the fibres to a certain extent, as well as forming a more or less distinct layer internal to the layer of fibres.

The innermost layer of the stem is a thin, ciliated, coelomic epithelium. It is not easy to distinguish this layer from the layer of muscle-cell bodies underlying it, except by the fact that the epithelial nuclei are usually flattened longitudinally, whereas the nuclei of the muscle-cells are usually round or pear-shaped. In the material available it is not possible to see whether the cilia of this epithelium are uniformly distributed or are in two main columns, one beating distally and the other proximally to maintain a circulation within the lumen.

The disk. The tissues of the disk are supported by a rosette of 5 calcareous plates (fig. 3) with the tube-foot lumen passing through a hole in the middle. The plates are embedded in continuations of the two outer connective-tissue layers of the stem, which are here mostly running longitudinally to insert at the epithelium of the disk; some of the fibres, however, pass in and out of the fenestrae of the plates to bind them together and keep them in place. The distal edges of the plates are scalloped and the upper surfaces are ridged: these excavations contain nerves arising at the nerve-ring, which lies against the proximal side of the rosette. The nerves turn in under the distal side of the rosette to become the sub-epithelial plexus of the disk. On their proximal inner surfaces the plates are excavated to form a cradle for the complete ring of flattened stem spicules, mentioned above.

After the tube-foot lumen has passed distally through the central hole in the calcareous rosette it narrows somewhat before terminating 20 to 25 μ from the surface of the disk. At approximately the place where the narrowing starts, which is also at the level of the complete ring of flattened stem spicules, muscle-fibres of the disk levator system originate at the connective-tissue sheath, pass into the lumen end distally, and insert at the sheath again on the inside surface of the diaphragm. In this set of muscles it is easy to identify the nucleus belonging to each individual fibre, which is contained in a small swelling of cytoplasm, generally near the distal end of each fibre.

The epithelium (figs. 5; 8, B) is remarkably uniform in thickness (about 17 to 20 μ in an adoral tube-foot) and structure over its entire extent. The main feature which characterizes it is the presence of muscle-fibres, quite regularly spaced every 3 to 6 μ, originating at the basement membrane and inserting at the external cuticle. Each fibre is about 2 μ in diameter, fanning out and breaking up into 5 or 6 component fibrils at each end, and has its own oval nucleus in a swollen region of cytoplasm about two-thirds of its length towards the cuticle. Scattered unicellular mucous glands are also present. These take up only faintly the light green of Masson and the red of mucicarmine, but show quite marked metachromasia with toluidine blue, &c. The third recognizable component of the epithelium is a series of ciliated cells, rather sparsely placed every 15 to 20 μ. As far as can be seen, the cilia arise singly from each cell. It has not been possible to determine whether these are motile cilia or sensory processes.

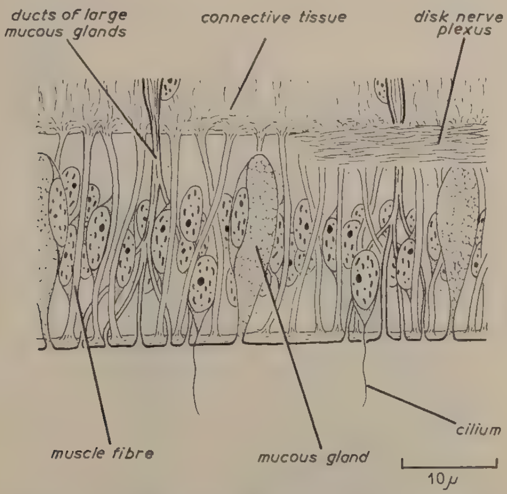

FIG. 5. Drawing of a vertical section through the epithelium of the disk of a suckered tube-foot of *C. cidaris*. The sub-epithelial nerve-plexus ramifies between groups of the proximal ends of the epithelial muscle-fibres and the ducts of the large mucous glands: for this reason it is shown only on the right.

Although not strictly belonging to the disk epithelium, one other component of importance occupies the disk: in addition to the single-celled mucous glands of the disk epithelium, there is a further series of single-celled glands aggregated into groups of up to 15 cells, the main bodies of these cells lying between the disk epithelium and the distal face of the calcareous rosette (figs. 3; 8, A). The glands send their ducts towards the face of the disk in groups, but within the epithelium the ducts separate and open through the cuticle singly.

The peristomial tube-feet

The stem. This is very similar to the stem of the suckered tube-foot. The same basic component layers are present, the chief differences in detail being as follows. First, in non-narcotized material it is evident that very much less puckering of the external ciliated epithelium occurs (fig. 6). It is clear from other evidence, such as the absence of ampullae for these tube-feet, that very little protraction can take place. The relative thickness of the retractor muscle-layer, however (it is only slightly thinner than that of the suckered tube-foot), suggests that postural movement relative to the test is important, as one would expect in an organ whose chief function is probably sensation, so some provision for crumpling in some of the layers must be made. This is particularly reflected in the connective-tissue layer. Here, only two layers are recognizable: an inner circular and an outer mainly longitudinal. The latter layer is dense internally but becomes somewhat spongy towards the outside. This spongy component could most likely distort to take up any differential contraction due to bending; in it, possibly to help retain the cylindrical shape, many small

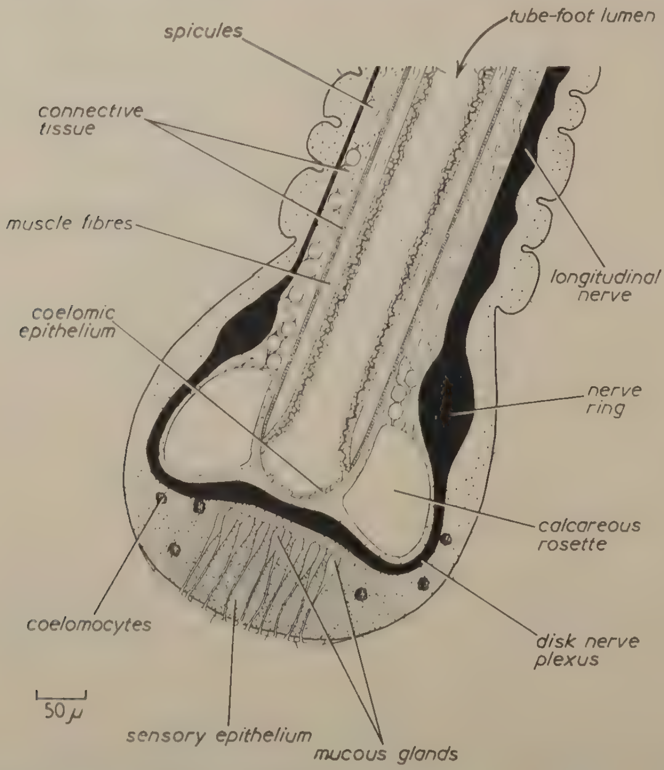

FIG. 6. Diagrammatic longitudinal section of the distal end of a peristomial tube-foot of *C. cidaris*. The section has been taken through the longitudinal tube-foot nerve (shown on the right of the stem). The sensory epithelium is shown densely stippled.

curved spicules are embedded, as in the comparable layer of the suckered tube-foot; the spicules overlap on one side and leave a gap for the longitudinal tube-foot nerve on the other, as before. Distally, at the level of the nerve-ring, the spicules form a complete ring and are here packed closer together, though they do not flatten out appreciably. The sub-epithelial nerve-plexus of the stem is thicker throughout, and the longitudinal nerve is particularly well marked.

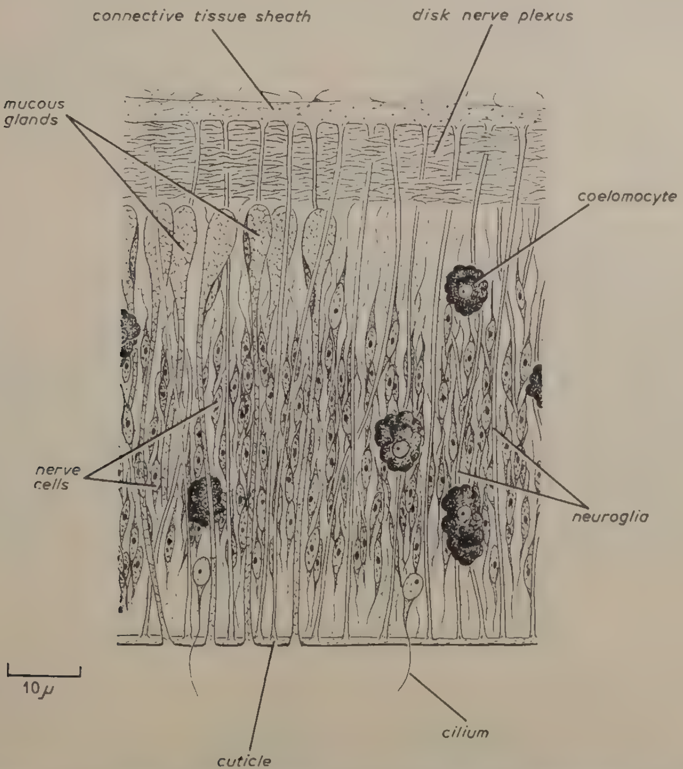

FIG. 7. Drawing of a vertical section through part of the sensory epithelium of a peristomial tube-foot of *C. cidaris*.

The disk. The disk (fig. 6) is supported by a ring-like calcareous rosette which is much smaller than in the suckered tube-foot, and which lacks the fluted edge. It is apparently formed of one single calcite element. As usual, the lumen protrudes distally through the central hole in this ring, though it does not extend much beyond the distal side of the rosette. Here there are no muscle-fibres representing a disk levator system: instead, the ciliated coelomic epithelium lines the distal end of the cavity. The disk has a much thickened epithelium distally (fig. 8, c). The chief component of this layer is a large number of closely-packed, elongated cells with oval nuclei, some of whose processes can be followed across the epithelium from the thickened cuticle towards the sub-epithelial nerve-plexus, which is particularly well developed.

These cells are probably sensory nerve-cells, for reasons which will be discussed later. A large number of connective-tissue fibres are also present. These can probably here be termed neuroglia. They traverse the epithelium, but pass across the nerve-plexus to originate at the continuation of the outer connective-tissue layer, here forming the 'cap' to the distal end of the tube-foot lumen. These neuroglial fibres in some ways resemble the epithelial muscle-fibres of the suckered tube-foot, but they are very much thinner (between $\frac{1}{2}$ and $1\frac{1}{2}$ μ as opposed to 2 μ) and take up the xylidene red of Masson and the red stains of Mallory and Azan rather less intensely.

The third recognizable component of the disk epithelium is a large number of coelomocytes. These appear a dull green in a Masson preparation, and seem to consist of several droplets surrounding a central nucleus. Identical structures have been seen in the lumen of the tube-foot and in intervening tissue, but in the latter in less numbers than in the epithelium. Fourthly, some short (3 to 4 μ) cilia emerge from the disk surface, as in the suckered tube-foot, and again it has not been possible to identify these as sensory or motile. Lastly, towards the inner margin of the epithelium, almost against the nerve-plexus, is a series of closely-packed single-celled mucous glands. The ducts from these glands open at the disk surface, and are exceedingly long and thin in consequence, an average gland being 80 μ long with a duct less than 1 μ in diameter.

THE HISTOLOGY OF THE TUBE-FEET OF *ECHINUS*

The suckered tube-feet

The stem. There is very little difference between this (fig. 10) and the stem of a *Cidaris* suckered tube-foot in the component layers and their relative thicknesses. The external epithelium and the coelomic epithelium lining the lumen are both ciliated, the latter being rather difficult to distinguish from the underlying layer of muscle-cell bodies. The connective-tissue sheath again

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FIG. 8 (plate). A, longitudinal section through the distal part of a suckered tube-foot of *C. cidaris*, roughly median. The sucker has become distorted during preparation, after decalcification of the skeletal rosette. See also fig. 3. All sections in this plate are from material narcotized in propylene phenoxytol, fixed in Heidenhain's 'Susa', and stained by Pantin's (1948) modification of Masson's trichrome technique. In all, the surface of the disk is towards the bottom.

B, longitudinal section of the diaphragm of a suckered tube-foot of *C. cidaris*, showing the insertion of the levator muscles at the connective-tissue sheath and the origin of the epithelial muscles on the other side of the same sheath. See also fig. 4.

C, longitudinal section through the distal part of a peristomial tube-foot of *C. cidaris*. See also figs. 6 and 7.

D, longitudinal section through the distal part of a suckered tube-foot of *E. esculentus*, showing particularly the groups of mucous glands embedded in the decalcified and hence distorted skeletal rosette. The nuclei of two of the disk-muscles in the diaphragm can be seen. See also fig. 10.

E, longitudinal section through part of the epithelium of D.

F, longitudinal section through part of the sensory epithelium of a peristomial tube-foot of *E. esculentus*.

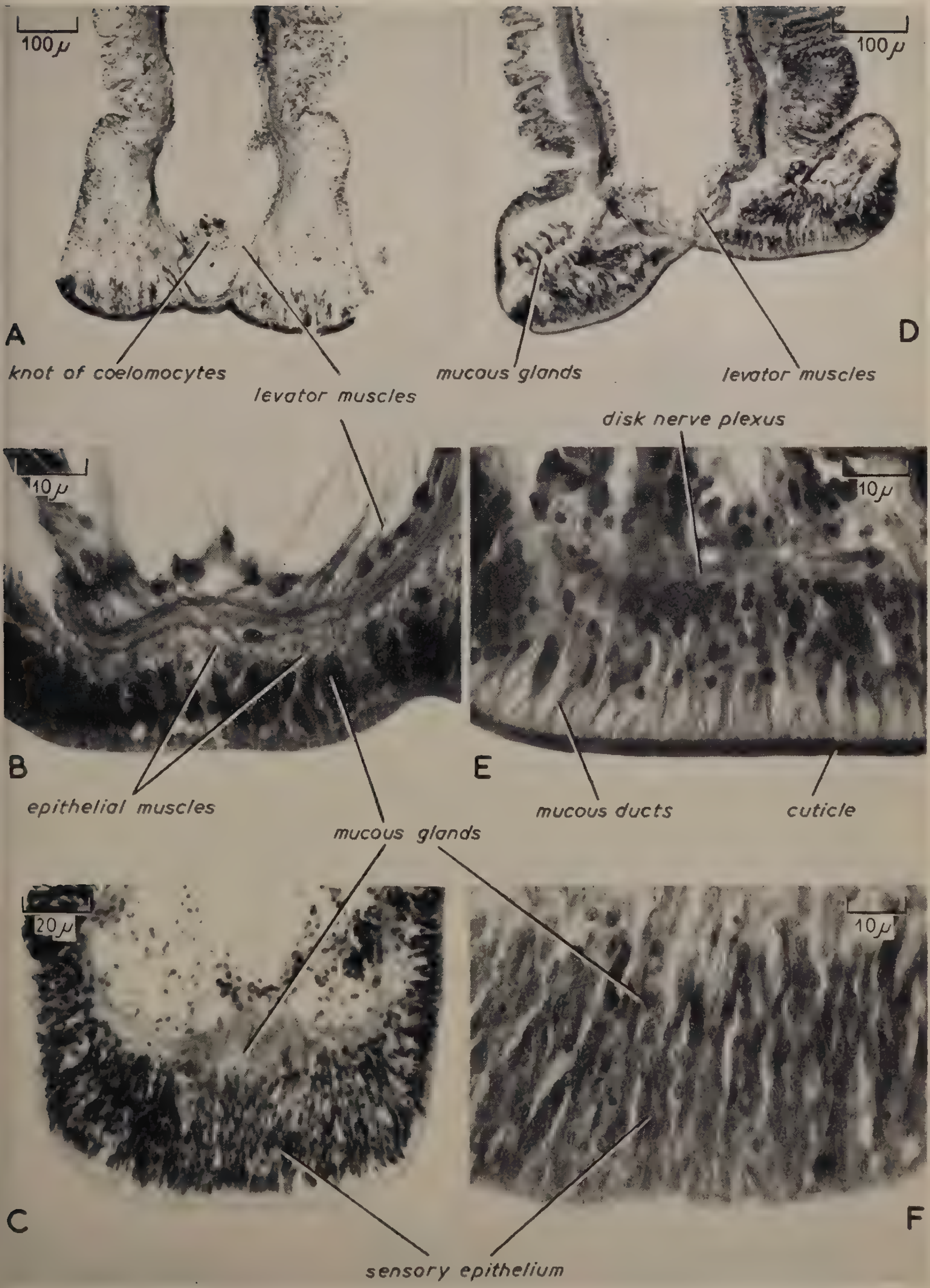

FIG. 8

D. NICHOLS

consists of three layers. The outer loose layer contains the calcareous spicules, which in this case are very numerous, *C*-shaped, almost thornless, and much smaller than in *Cidaris* (80 to 90 μ in length as opposed to 150 to 200 μ). They are not arranged in two longitudinal columns, but are scattered haphazardly in the outer connective tissue, and are absent only from the region of the longitudinal nerve.

The disk. Like that of the *Cidaris* suckered tube-foot, the disk is supported by a calcareous rosette (fig. 9, A). The main difference between the two rosettes is that here, although the edge is scalloped, the proximal surface is not channelled to take the distal branches from the tube-foot nerve-ring. There is a difference, too, in the nature of the spicular frame on the proximal side of the rosette. In this case there is no gradual flattening of the spicules at the distal end of the stem: instead the change is sudden, and the spicules of the frame are very much larger than those of the stem (350 μ in length); also, they are not bihamate, but shaped as in fig. 9, B. The spade-like flanges at each end overlap the adjacent spicules, while the fenestrae of the inner border provide pathways for the mainly longitudinal fibres of the connective-tissue sheath, which run distally towards the disk epithelium. The spicules of the frame form a complete interlocking ring, the 'psellion' of Lovén (1883), fitting into a cradle on the inner proximal side of the rosette, and are virtually embedded in the middle component of the connective-tissue sheath. In this case the elements of the frame are bound much more closely together than in *Cidaris*.

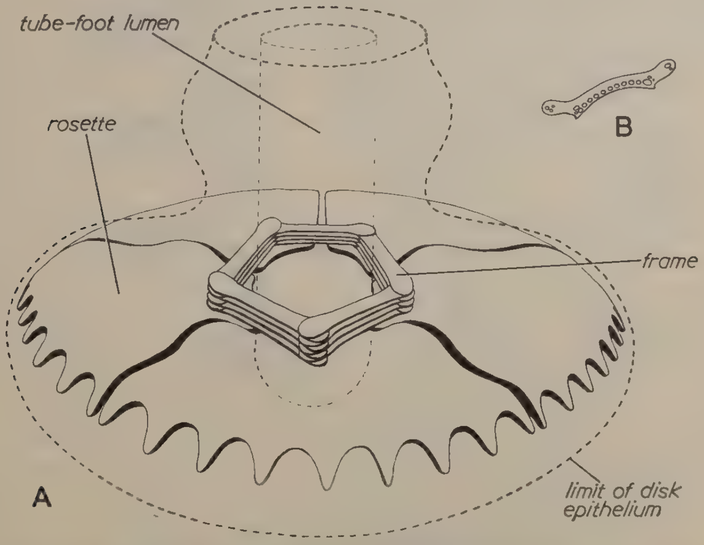

FIG. 9. A, perspective diagram to show the structure and arrangement of the skeletal elements in the disk of a suckered tube-foot of *E. esculentus*. The elements have been simplified for clarity. B, drawing of one spicule from the skeletal frame, showing the fenestrae through which the mainly longitudinal strands of connective tissue pass to the disk.

The muscle-fibres of the disk levator system (figs. 8, D; 10) originate at the connective-tissue sheath at the level of the skeletal frame, passing distally to insert at the inner wall of the diaphragm. There are over 100 of these fibres in a tube-foot of average size, and each has its nucleus in a cell-body close to its distal end.

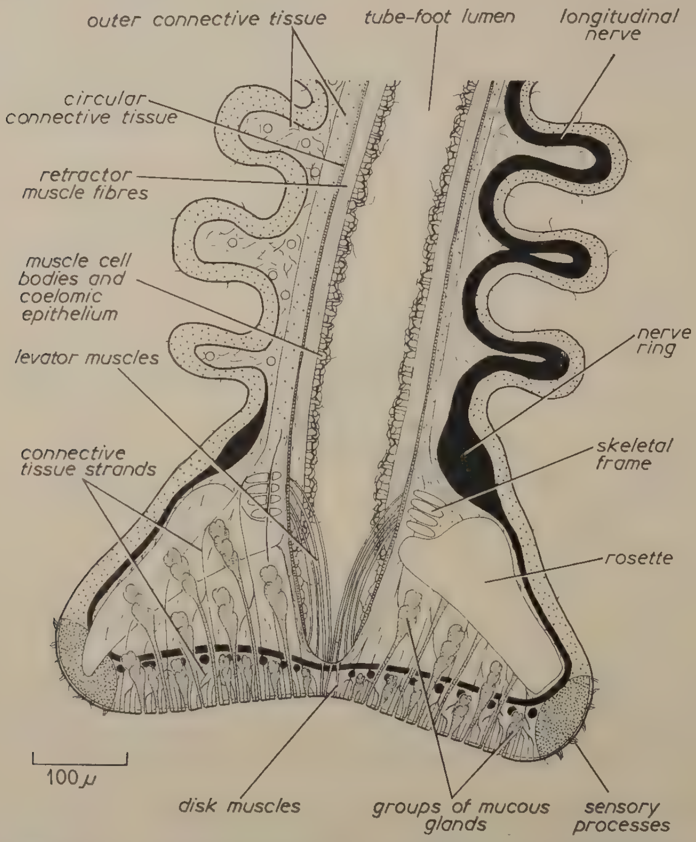

FIG. 10. Diagrammatic longitudinal section of the distal end of a suckered tube-foot of *E. esculentus*. The section has been taken through the longitudinal tube-foot nerve (shown on the right of the stem). On the right side of the disk the section has been taken through one of the indentations in the edge of the skeletal rosette, while on the left the section shows the passage of connective-tissue strands through the rosette and the position of some groups of mucous glands in the spaces of the rosette. A few only of the series of disk levator muscles have been included.

In the body of the disk itself there are 3 main non-skeletal structures. First, there are many single-celled mucous glands, aggregated into groups of between 10 and 20 to a group, and each sending a duct to the disk surface; the ducts of a group may lie together as they pass distally from the cell-bodies, but they separate eventually to pierce the disk cuticle at intervals of every 2 to 3 μ or so. Unlike the large glands of the *Cidaris* suckered tube-foot, some of these even lie within the framework of the skeletal rosette, so their length can be up to 175 μ; other groups of glands do not extend into the disk beyond the sub-epithelial plexus, while others extend beyond the plexus but not into the rosette. Secondly, there is a very prominent nerve-plexus in the disk. The nerve-ring gives off radial branches which pass over the proximal surface of the rosette and turn through the indentations in its scalloped edge to become the plexus of the disk. This plexus has two components: one in which the direction of the fibres is mainly radial, and a second, distal to it, in which the direction is mainly concentric; there are frequent connexions between the two, and both components of the plexus ramify between the branches of the connective tissue and the cell-bodies and ducts of the disk mucous glands. Thirdly, there are strands of connective tissue permeating the material of the disk. The fibres of the middle and outer connective-tissue layers of the stem become mainly longitudinal towards its distal end. Some of them pass through the skeletal frame and/or the rosette, running between the groups of mucous glands, past the sub-epithelial nerve-plexus of the disk, and give off many horizontal branches which eventually turn distally to insert at the disk cuticle. Smith (1937, 1947*a*) has described in detail the very similar arrangement of fibres in the tube-foot of the asteroids *Marthasterias* and *Asterias*, and there is no reason to think that the arrangement in *Echinus* is markedly different. Lastly, at the very centre of the diaphragm there are between 5 and 20 muscle-fibres originating at the connective-tissue sheath and inserting at the disk cuticle. The epithelium as such, i.e. the region of the disk distal to the disk nerve-plexus, is very much thicker in *Echinus* than in *Cidaris* (40 to 45 μ as opposed to 20 μ) and consists of the branches of the connective-tissue strands, groups of unicellular mucous glands, and ducts of others the bodies of which are embedded more deeply in the disk (see above).

The distal insertions of the connective-tissue strands are interesting. Proximally, the fibres take up the light green of Masson (or the aniline blue of Mallory) as normal, but about 1 to 2 μ from the cuticle the character of the fibre changes: they swell slightly and insert in a cup-shaped structure which takes up the xylidene red of Masson (or the red dyes of Mallory). In most cases the red-staining distal cup does not envelop the knob evenly on all sides: there may be a tail extending for up to 5 μ on the outer (centrifugal) side of the fibre. In transverse or in tangential sections (fig. 11) the arrangement is clearly seen. Passing from the cuticle proximally the following regions can be identified: just inside the cuticle the red distal regions of the fibres lie in the centres of irregularly shaped cells; interspersed among them are the ducts of the mucous glands, taking up the light green of Masson very faintly. Internal to this the green (or blue) regions of the fibres appear at their centres, and, moving inwards through the series, this part of the fibre becomes thicker until the red part is seen on the centrifugal side only. Internal to this, the fibres lose the red surround, become somewhat thinner (scarcely more than $\frac{1}{2}$ μ thick), and lie among the continuations of the mucous ducts, which in this region are still very thin-walled. At this level the cell-walls of the connective-tissue cells are not easy to see. At this level, too, some nuclei of the mucous glands appear, and the cytoplasm of a few of the glands becomes thicker; these are the distal parts of the cell-bodies of the small glands which occupy the true disk 'epithelium', i.e. the region distal to the nerve-plexus. Here also, some nerve-fibres are seen.

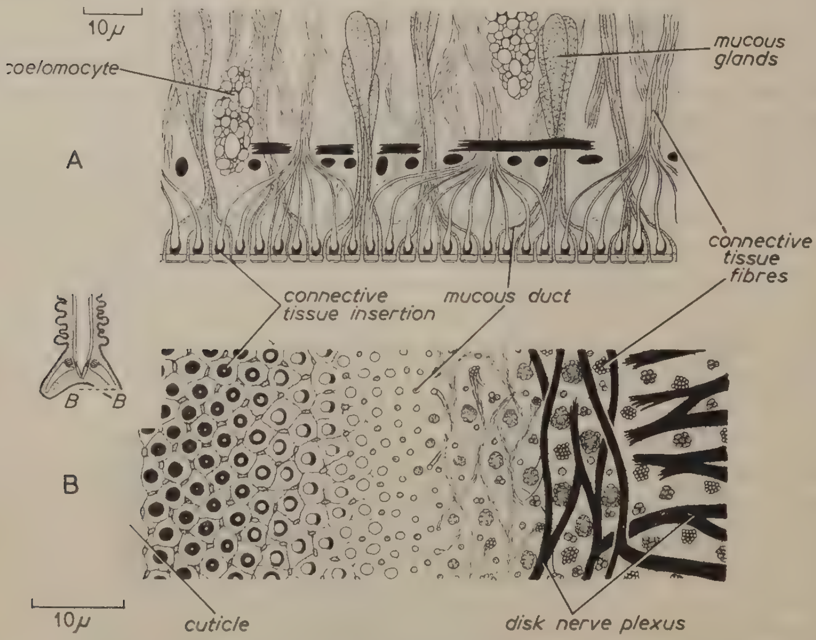

FIG. 11. A, semi-diagrammatic drawing of a longitudinal section through part of the disk epithelium of a suckered tube-foot of *E. esculentus*, showing the distal insertion of the connective-tissue fibres and the ends of the single-celled mucous glands. B, semi-diagrammatic tangential section through A, taken through the line *BB* on the inset diagram. The pathways of the nerve-plexus (on the right) and the distal, red-staining parts of the connective-tissue fibres (on the left) are drawn in black; green-staining parts of the connective tissue when cut in transverse section are left blank; mucous glands and their ducts are densely stippled. See description in the text (p. 171).

At the next level the green connective-tissue fibres mostly turn through a right angle, to lie parallel to the disk surface; this is the region just below the main nerve-plexus of the disk where the connective-tissue strands fan out before running down perpendicularly to insert at the disk surface. Among the groups of fibres are the cell-bodies of the smaller mucous glands and the ducts of the larger ones, together with some nerve-fibres. Next, the connective-tissue fibres become aggregated into groups, and, where necessary, turn through a right angle to become the main connective-tissue strands which connect up with those of the stem sheath. At the level where the fibres aggregate they are interwoven by the distal (concentric) component of the sub-epithelial nerve network of the disk, some of whose fibres can be seen passing among the mucous glands and possibly terminating at them. In many places large elongated nuclei, 5 to 6 μ long and 2 to 3 μ wide, lie close to the fibres, even embedded between them with their axes parallel to the direction of the fibres, and these may well be the nuclei of nerve-cells, possibly sensory. Some fibres from this layer can be seen passing inwards (proximally) and turning through a right angle to connect with fibres of the inner nerve-plexus, where the main direction of the fibres and their elongated nuclei is radial.

There is a heavily nucleated ring at the periphery of the disk (fig. 10). The cells in this region have processes passing distally to pierce the cuticle in groups of between 3 and 6, and other processes running proximally to enter the sub-epithelial nerve-plexus. These facts suggest that this is an aggregation of sensory nerve-cells. As far as can be seen, there are no other processes piercing the disk cuticle comparable to the cilia of the *Cidaris* tube-foot disk.

The peristomial tube-feet

The stem. The main difference between the stems of these tube-feet and those of the other tube-feet described in this paper is the complete absence of calcareous spicules in the connective-tissue layer. Coupled with this is the absence also of an outer, diffuse layer to the connective-tissue sheath (the layer in which, in the other tube-feet, the spicules are embedded). Otherwise, there is little basic difference in the structure of the stem.

The disk. Here again the disk is supported by a calcareous rosette. In this case, too, the rosette appears to be formed of a single element. This is oval, the long axis being tangential to the edge of the mouth. The central hole is also oval and carries the distal end of the tube-foot lumen (fig. 12). There are no spicules present in the disk in the form of a frame on the proximal side of the rosette. Gordon (1926), describing the development of the closely-related *Psammechinus* (= *Echinus*) *miliaris*, remarks that the only calcareous element to be formed in the disk of its primary peristomial tube-feet is a single, rather dense, oval primordium.

As is to be expected, nervous tissue is very much in evidence here, as in *Cidaris* peristomial tube-feet. The longitudinal nerve, the sub-epithelial plexus of the disk, and the nerve-ring are all conspicuous, the ring being somewhat irregular in shape. The disk epithelium has a high proportion of sensory nerve-cells, the distal processes of which run to the inner border of the cuticle while the proximal processes run towards the sub-epithelial nerve-plexus, where some can be seen joining it. Many neuroglial cells support the thick epithelium, just as in the comparable place in *Cidaris*, originating at the connective tissue in which the rosette is embedded and inserting at the disk cuticle. The third element of the epithelium is a large number of mucous glands; they are very long and thin (3 to 5 μ is the average width at their widest point) and extend from the cuticle to the distal border of the nerve-plexus. In my preparations these glands failed to take up the light green of Masson, but showed up well with Southgate's mucicarmine and Mayer's muchaematein. The sensory epithelium of this tube-foot is unique in that it extends not only over the entire distal surface of the disk but over a large part of its proximal surface as well. Sensory nerve processes can even be seen entering the distal parts of the nerve-ring; the mucous glands, however, do not extend beyond the edges of the rosette.

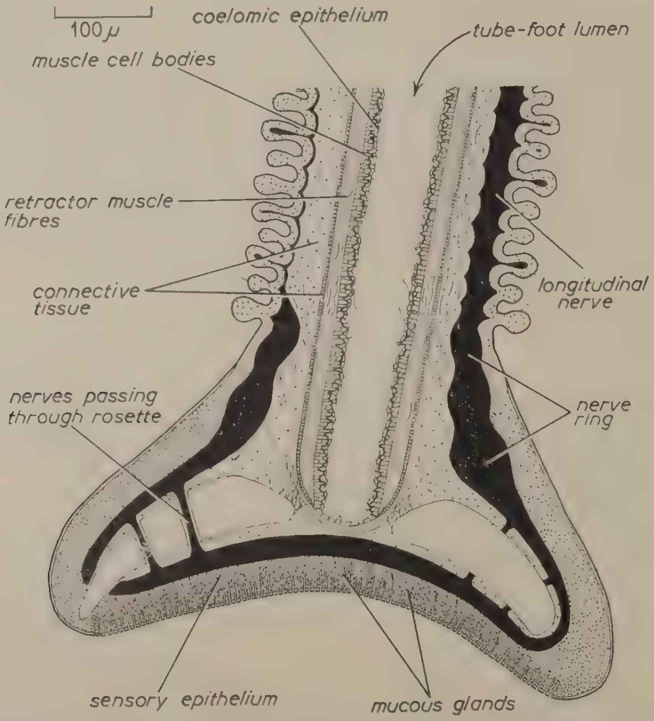

FIG. 12. Diagrammatic longitudinal section of the distal end of a peristomial tube-foot of *E. esculentus*. The section has been taken through the longitudinal tube-foot nerve (shown on the right of the stem). On the right side of the disk the section has been taken through one of the indentations in the edge of the rosette, while on the left the section shows the passage of nerve-tissue through two of the fenestrae of the rosette. The sensory epithelium is shown densely stippled.

DISCUSSION

Four different types of tube-foot of two different urchins have been described in this paper. Reduced to structures which are common to all of them (disregarding some secondary losses), the basic pattern of echinoid tube-foot histology is as follows. The stem consists of a sheath of connective tissue with embedded spicules, to preserve the shape and maintain a constant diameter, a longitudinal retractor muscle, and an inner and outer ciliated epithelium, the one continuous with the epithelium lining the rest of the water-vascular system and the other continuous with the epithelium covering the animal and all its appendages except teeth. Underlying the external epithelium is a nerve-plexus which is expanded on the oral side of the tube-foot into a longitudinal nerve. At the distal end the external epithelium and the connective-tissue layer are expanded to form the disk. Here, the calcareous spicules in the connective tissue are greatly enlarged, and the epithelial cells of the distal face are modified to, or replaced by, the following types of cell: mucous gland, sensory, and supporting.

An animal equipped with hydrostatic organs of this basic type would be able to use them for respiration, sensation, and, to a certain degree, locomotion and anchorage, by utilizing a sticky secretion from the terminal glands. Clearly, considerably greater efficiency would be obtained if some device for raising the centre of the disk surface could be incorporated into the design. This has happened in the suckered tube-feet of the urchins described in this paper, by developing a disk levator system of muscles; the stem spicules are enlarged at the distal end of the stem to act as an anchor for these muscles. In *Cidaris* further efficiency is brought about by the replacement in the disk epithelium of some supporting fibres by muscle-fibres, presumably to ensure an adequate ejaculation of mucus on to the disk surface during adhesion. In addition, larger mucous glands, in groups, are embedded in the material of the disk close under the enlarged spicules forming the rosette, and when the diaphragm is raised they are presumably compressed against the distal face of the rosette, and thus their efficient discharge is ensured. For detachment, there are no radial muscles on the inner face of the diaphragm, such as Smith (1947*b*) describes for the tube-feet of the asteroid *Asterias rubens*; instead, it is likely that the disk skeleton, absent in asteroids, plays a part here by transmitting the pull of the retractor muscles of the stem to the outside edge of the disk, so that when the levators relax and the retractors contract, the result is to drop the diaphragm and raise the edge of the disk, thus releasing the tube-foot's hold.

The suckered tube-foot of *Echinus* has further improvements on the cidaroid plan. First, the spicular frame is bound together more closely and more strongly, presumably so that the levators are more effective. One would expect, as a result, a stronger sucker action, and this is certainly confirmed by observation of the living animal (p. 160). Secondly, all the mucous glands are aggregated into groups, even those on the distal side of the disk nerve-plexus, and so they can all compress against each other or against the skeletal rosette for efficient discharge when the diaphragm is raised, without the need of special epithelial muscles. Thirdly, the muscle-fibres of the disk, no longer required for mucous discharge, are restricted to that part of the epithelium where they can be most effective in augmenting the task of the levators, namely, at the centre of the diaphragm. Lastly, the sense-cells appear to be concentrated into that part of the disk where they will be most effective, that is, at the periphery, which is usually the region first to touch the substratum during locomotion.

As far as the peristomials are concerned, the most noticeable feature of those of *Cidaris* is the shape of the skeletal rosette, here reduced in diameter and forming merely a knob for the support of the rather blunt disk. Most of the cells in the epithelium have an appearance consistent with their having a sensory function (p. 167), possibly both tactile and chemoreceptive. The terminal mucous glands are retained, presumably to ensure that the tube-foot adheres to an object long enough to test its suitability or otherwise as food. In the living animal these tube-feet certainly do attach temporarily to the substratum over which the animal is moving and detach as though breaking the adhesion of a sticky secretion. In the peristomials of *Echinus* the disk is very much more extensive, and here again its surface is made sticky for the adhesion necessary to taste the substratum. The unusual feature of these is the extent of the sensory epithelium, which here occupies the proximal sides of the disk as well as its distal face, so that the actual sensory area is 5 or 6 times that of a *Cidaris* peristomial tube-foot.

On the apical side of the test the tube-feet of both *Cidaris* and *Echinus* have very much thinner walls and tend to have less developed suckers; close to the apical disk some even become completely blunt. It is unusual, of course, for these urchins to attach by their apical sides, and so the tube-feet in this region are to be regarded mainly as respiratory organs, though the possibility of their being important in excretion also must not be overlooked. As respiratory organs they may transport oxygen through the water-vascular system to other parts of the body, or oxygen may diffuse through the thin walls of the ampullae into the perivisceral coelom. In *Echinus* one region of the body coelom, that surrounding the Aristotle's Lantern and called the perioesophageal coelom, has no ampullae opening into it. It is not surprising, therefore, that special gills protrude from it through the peristomial membrane to supply just this cavity and the important complement of jaw-muscles it contains with oxygen. In the case of *Cidaris* the lack of disks and general simplification is evident in rather more of the apical tube-feet than in *Echinus*; this suggests that oxygenation of the perivisceral coelomic fluid by them is even more important. Probably coupled with this is the fact that there are no peristomial gills comparable to those of *Echinus*. There are, however, blind-ending, somewhat lobulated sacs, the organs of Stewart (1879), extending into the perivisceral coelom, whose lumina are continuous with the perioesophageal coelom. They are very thin-walled, and it therefore seems likely their function is to cope with the respiratory needs of the perioesophageal coelom and the jaw-muscles in it by obtaining oxygen from the apical tube-feet through the perivisceral coelom. Although Stewart did suggest a respiratory function for them, he thought they acted in the opposite way: he considered that the jaw-chamber probably communicates with the surrounding water near the tips of the teeth; then, since the volume of the jaw-chamber could be increased by raising the compasses of the Aristotle's Lantern when the transverse muscles contract, he considered that it is the interior of the lobulated organs which act as gills, passing the oxygen they obtain in this way to the body coelom. It seems unlikely that the perioesophageal coelom is in contact with the outside water, and so the movements of the Lantern compasses probably serve to pump coelomic fluid in and out of Stewart's organs for oxygenation from the rest of the coelom, not, as Stewart thought, to pump sea-water in and out of them.

In the clypeasteroid sea-urchins, e.g. *Echinocyamus* (Nichols, 1959*b*), the transformation from suckered tube-feet in the oral and ambital parts of each ambulacrum to respiratory tube-feet in the apical parts is sudden. Otherwise, the division of labour is no more marked than it is in the regulars. The walls of the respiratory tube-feet in *Echinocyamus* consist of little more than the external and coelomic epithelia, and, of course, the ampullae are entirely lacking. The other two types of tube-foot, suckered and buccal sensory, however, closely resemble their equivalents in the regulars. The suckered tube-foot of *Echinocyamus* (Nichols, 1959*b*, fig. 2) has the same component layers in the stem: a disk levator system of muscles, a series of mucous glands opening on the disk, a sensory ring at the edge of the disk, and possibly a series of muscle-fibres in the epithelium of the diaphragm, though these may be non-elastic supporting fibres. There are disk muscles, as in *Cidaris*, though here they functionally replace the skeletal rosette during detachment rather than act as mucous gland squeezers. The buccal tube-feet of *Echinocyamus* consist of little more than nerve-tissue, with supporting neuroglia traversing it, just as in the peristomials of regulars, though there are apparently no mucous glands. Thus, except for very minor points of histological detail, the suckered and buccal sensory tube-feet of *Echinocyamus* are virtually miniature copies of their equivalents in the cidaroids, which are probably ancestral to them; the tube-feet of the dorsal surface would seem to be a functional improvement on those occupying a similar position in the regulars, particularly when considered in relation to the ciliary currents of the external epithelium in their vicinity (Nichols, 1959*c*).

Sea-urchins of the order Spatangoida show much more division of labour in their tube-feet than either the regulars or the clypeasteroids (Nichols, 1959*a*). Except for the respiratory tube-feet on the dorsal surface, which are similar in arrangement and basic histology to those of *Echinocyamus*, and the sensory tube-feet occupying mainly the ambital parts of each ambulacrum, the tube-feet of a spatangoid are mainly mucus-producing organs, their design differing according to the function to which they put the secretion. In general, their disks bear numerous papillae on the distal face or round the edge, and normally the skeletal elements of the disk are retained, not as a rosette but as skeletal rods within the papillae; the retractor muscles of the tube-foot stem insert at the centripetal ends of these rods, just as the retractors of the regular echinoid tube-foot insert at the centripetal edge of the elements of the rosette. The disk epithelium is extended on to the distal side and/or round the tips of the papillae; the single-celled mucous glands are, of course, retained and so are the disk muscle-fibres in those cases where a forcible discharge of mucus is required (Nichols, 1959*a*, fig. 6).

It has been remarked above (pp. 160, 175) that *Cidaris* is much less dependent on the action of its suckered tube-feet for locomotion than *Echinus*. In *Cidaris* the spines are the main locomotory organs, the tube-feet acting as anchors during this activity, so that the force they exert is probably mainly perpendicular to the test. In *Echinus*, on the contrary, movement is principally brought about by contraction and bending of the tube-feet. Although no subdivision of the tube-foot retractor muscles of *Echinus* has been detected (unlike the suckered tube-foot of *Echinocyamus*, where the muscle is arranged in 4 longitudinal columns), differential contraction does take place while the tube-foot is bending to a new site and probably also when the sucker is attached. But in *Cidaris* very much less bending of the tube-feet is seen, even when the disks are free. So it is not surprising that the area of the test enclosed by the base of the suckered tube-foot of *Echinus* is greater than that of *Cidaris* to obtain greater bending moment (compare fig. 2, B, E). A comparable situation occurs in some recent spatangoids, in which the tube-feet whose main extension is perpendicular to the test, e.g. those of the dorsal part of the anterior ambulacrum of *Echinocardium cordatum* (Nichols, 1959*c*, fig. 27), have a smaller area of attachment to the test than those which move at all angles to the test, e.g. those from the phyllodes of *E. cordatum* (Nichols, 1959*c*, fig. 28).

On the oral side of the perradial pore of each pair in both *Cidaris* and *Echinus* there is a groove which emerges at the test surface and leads to a furrow running along the oral side of the projection between the pore-pairs (fig. 2, B, E). This furrow cradles that branch of the radial nerve which leads to the tube-foot, the longitudinal tube-foot nerve originating from the part of this branch which lies between the two pores. The retractor muscle-fibres of one side of the tube-foot originate at the oral surface of the ridge or hemispherical mound between the two pores of a pair (fig. 2, B, E), while those of the other side originate on the wall of the groove or depression which marks the aboral limit of the tube-foot base. This arrangement helps to clarify the function of the ornamentation in the region of the pore-pairs in the Cretaceous spatangoid urchin *Micraster*; in this burrowing form those pore-pairs which bear extensile tube-feet (in the phyllodes round the mouth, in the dorsal part of the anterior ambulacrum, and in the sub-anal region) all have bulges of calcite between the individual pores (Nichols, 1959*c*, figs. 41, 43, 45), which is not so in such recent spatangoids as *E. cordatum*, *Spatangus purpureus*, and *Brissopsis lyrifera*: in these the pore-pair is usually replaced by a single pore and the region of the test for the attachment of the tube-foot retractor muscles surrounds this pore on all sides (Nichols, 1959*c*, figs. 27 to 31).

Of the three grooves into which the pores bearing the peristomial tube-feet of *Echinus* are divided, two represent the remains of a pore-pair, which for some reason has become a single pore, while the third, that nearest the oral side, holds the longitudinal nerve, important in a tube-foot whose function is mainly sensory.

The fullest account of the tube-foot of *Cidaris cidaris* (= *Dorocidaris papillata*) is that of Prouho (1887), who describes the histology of an adoral suckered tube-foot as representative of the entire sub-equatorial portion of the test, including (incorrectly) those emerging from the peristome. Though recognizing the calcareous rosette as homologous with that of the *Echinus* tube-foot disk, and remarking that the distal members of the series of stem spicules form a complete ring, he does not recognize this ring as homologous with the frame of the *Echinus* tube-foot. He recognizes a series of muscles independent of the longitudinal retractors which insert at the diaphragm, the series later called levators by Smith (1947) in asteroids, and describes their action. Apparently Prouho does not recognize the presence of mucous glands in the disk epithelium. He does describe the mainly longitudinal connective-tissue fibres passing through the spaces in the calcareous rosette, but is apparently unaware that the sub-epithelial nerve-plexus originating at the nerve-ring of the tube-foot is cradled in grooves on the proximal side of the rosette. Because of this, he does not describe those elements of the nervous system distal to the point where the disk nerve sinks into the grooves in the rosette.

Hamann (1887) has previously described the histology of the *Echinus* suckered tube-foot. He figures a skeletal rosette supporting the disk, and includes the connective-tissue fibres which permeate the skeletal material, and also an irregular series of spicules at the place where the frame lies. He shows some of the mucous glands of the disk, but not those embedded in the rosette. He does not recognize a series of levator muscles separate from the longitudinal series, and therefore does not recognize the function of the spicular frame. Of the nervous system, he describes the main tube-foot nerve-ring as a *Nervenpolster* (nerve pad), and is apparently not aware that the main direction of the fibres changes here to run round the circumference of the foot. At the periphery of the disk, however, in the position of the ring of sense-cells, he describes a marginal nerve-ring, which apparently gives off the disk nerve-plexus, though this is not clear; of the disk nerve-plexus, he shows fibres in one direction only (radial).

A far more accurate picture of the two parts of the disk skeleton is given by Lovén (1883) for the closely-related *Strongylocentrotus* (= *Toxopneustes*) *dröbachiensis*. He shows the frame of flattened spicules, which he calls the psellion, on the proximal side of the rosette, with the levator system originating from a position close to them; he illustrates each element of the frame as a single, annular spicule in this urchin, whereas in *Echinus* each is a series of slightly curved rods arranged in a ring. Gordon (1926) has shown that during the development of the suckered tube-foot of *Psammechinus* (= *Echinus*) *miliaris* the elements of the frame arise as separate entities, but as the imago grows they 'appear to fuse together to form a somewhat hexagonal ring'.

I wish to record my indebtedness particularly to Dr. A. J. Southward for presenting me with one of his living specimens of the rare *Cidaris*, with details of its locality; to the Director and staff of the Marine Biological Laboratory, Plymouth, for providing facilities for some of the work; and to Miss E. Collins and Mr. J. A. Haywood for technical assistance. My thanks are specially due to Mr. Duncan Heddle for much useful discussion, and for reading the manuscript of this paper, and to Prof. Sir Alister Hardy, F.R.S., in whose Department at Oxford most of the histological work was carried out.

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The Iridophores of the Echinoid *Diadema antillarum*

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With one plate (fig. 1)

SUMMARY

The blue pattern seen when the skin of *Diadema antillarum* is viewed by reflected light is due to iridophores. The quality of their blueness varies with the background provided by neighbouring chromatophores.

Microdissection reveals that they consist of gelatinous plates contained in a fibrous and cellular capsule. The study of fixed preparations shows that the plates are sheathed and that their structure is greatly altered by fixation which distorts, disrupts, or dissolves them.

The structure of the iridophores bears little resemblance to the description of the so-called 'eyes' of *D. setosum* given by Sarasin and Sarasin, with which they appear to correspond.

Examination of their optical properties indicates that Rayleigh scattering by the colloidal contents is the most likely cause of their colour.

INTRODUCTION

THE striking colour of the echinoid *Diadema antillarum* Philippi depends on three factors: ubiquitous black (provisionally identified as melanin) and red or purple (hydroxynaphthaquinone) pigments, interlaced with a brilliant blue pattern. The last is due to light reflected from structures in the skin, to which brief reference has already been made (Millott, 1953 *a*, *b*, *c*), though the ideas concerning the precise means whereby the colour is produced have needed revision.

Conspicuous blue spots appear also in the skin of other diadematids (Mortensen, 1940), but, apart from those of *D. setosum*, little has been written on their structure and on the means whereby their colour is produced. Sarasin and Sarasin (1887) have presented the most detailed existing description of the blue spots in a species from Ceylon, but it seems uncertain whether the species to which they refer is *D. setosum* (Leske), since before 1904 this species appears to have been confused with *D. savignyi* (Audouin) Michelin (see Mortensen, 1940, p. 259). Subsequent brief references to these structures in this and other diadematids by Cuénot (1891, 1948), Mortensen (1940), and Millott (1953 *a*, *b*) raise doubts as to the adequacy of the Sarasins' account.

Döderlein (1885) believed the blue areas to be luminous, while Sarasin and Sarasin believed they were eyes. The evidence is wholly inadequate, and in the case of *D. antillarum* the structures appear to be iridophores (Millott, 1953 *a*, *b*), a view which has been fully substantiated by subsequent

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study. They may perhaps function like a tapetum in the highly organized photoreceptive surface that covers the test (Millott, 1960).

DISTRIBUTION OF THE IRIDOPHORES

When the living skin is examined microscopically by light reflected from a microscope point-light, an elaborate and exceedingly beautiful pattern of brilliant blue lines and spots appears against the red and black background of the skin. We have studied their distribution more closely in individuals that are about half-grown (roughly 3·5 cm across the ambitus).

Their distribution follows mainly that of the white pattern which is developed especially in young urchins, when the chromatophore pigment is concentrated (Millott, 1952) so that the iridophores are largely interambulacral and in greater number aboral to the ambitus.

They surround the periproct in a position corresponding to the inner border of the white ring that forms in this area, being arranged predominantly in a single row to form a discontinuous line that passes round the madreporite (fig. 1, A). Here and there the row is double.

In the middle line of each interambulacrum a band of iridophores extends toward the ambitus along each margin of a shallow depression or gutter in the test (fig. 1, A), which is conspicuously white when the chromatophore pigment is concentrated. The bands are irregular owing to the uneven spacing of the iridophores, which may be isolated or packed in a row (fig. 1, D). Aborally each band approaches the ring around the periproct, but it does not join it. As the bands pass to the ambitus they diverge sharply where the

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FIG. 1 (plate). A, the distribution of the iridophores aboral to the ambitus shown by a portion of the test from which most of the spines have been removed. The iridophores, seen by reflected light, appear as discontinuous white lines. The irregular white areas are the cut surfaces of the spines or test. The specimen is partly light-adapted so that some of the white pattern, formed when the chromatophore pigment is concentrated, is still visible between the rows of iridophores which run radially down the middle of each interambulacrum.

B, C, D, the effect of spreading skin pigment (p. 184) on the appearance of the iridophores which lie alongside the white lines developed in darkness in the gutter-like depressions of the interambulacra.

B, an early stage; the iridophores are barely distinguishable along the right margin, while those outside the white areas are well defined. The punctate or stellate black bodies in the white area are chromatophores.

C, the same area after longer exposure to light. Pigment has encroached on the white area (particularly along the right margin) in the processes of chromatophores underlying the iridophores, which are now more easily distinguished.

D, a later stage from an area aboral to that shown in B and C, where more chromatophore pigment has spread beneath the iridophores on both sides so that they are now clearly defined and appear blue.

E, a cluster of iridophores showing the dispersion of melanophore pigment in fine processes around them.

F, a horizontal section of an iridophore fixed in Carnoy ($1\frac{1}{2}$ h), stained by the PAS technique, and showing the thin plates separated by droplets which are strongly PAS-positive (see p. 190).

G, a vertical section through an iridophore of a young individual, fixed in Champy ($2\frac{1}{2}$ h), stained in Heidenhain's iron haematoxylin and light green. For description see p. 188.

H, a horizontal section through an iridophore fixed in Champy ($2\frac{1}{2}$ h), stained in Weigert's haematoxylin and light green. For description see p. 190.

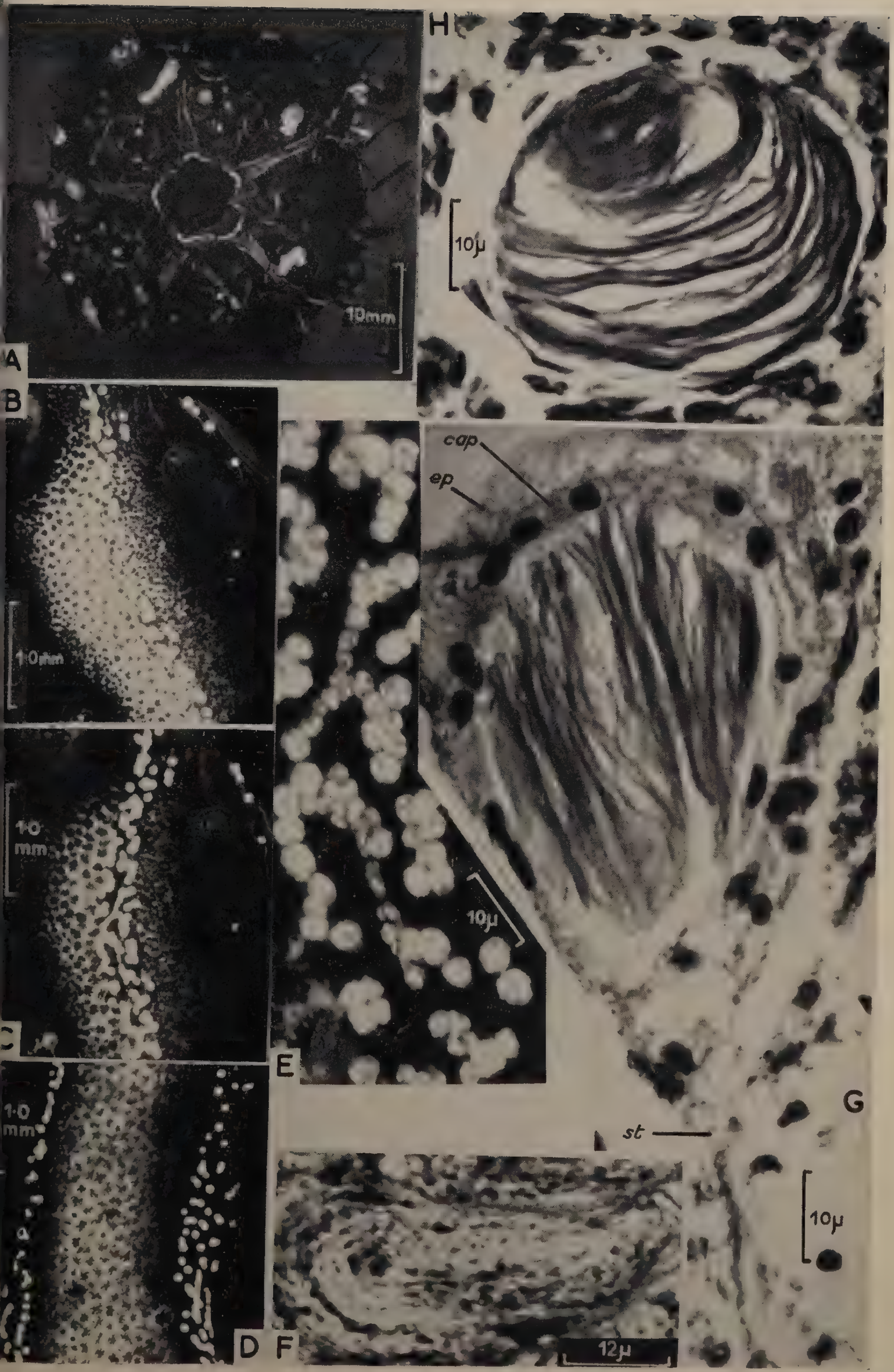

FIG. 1

N. MILLOTT *and* B. M. MANLY

gutter bifurcates to form a figure resembling the head of a lancet arch, which when devoid of pigment forms such a conspicuous and characteristic pattern in the interambulacrum. Each band of iridophores follows the outer margin of this figure, becoming thicker and irregularly branched, sometimes to such an extent that the ramifications form a lace-like network (fig. 1, E).

Here the iridophores are more densely packed into ridges or cushion-like masses, superficially resembling the faceted cornea of a compound eye, a likeness which, from the accounts given by the Sarasins, seems to have its counterpart in *D. setosum* and to have been partly responsible for their suggesting that these structures are eyes.

Some of the branches from the bands are more extensive, passing between the bases of the adjoining primary spines, around which they extend to form discontinuous rings composed mostly of single rows, thickened irregularly to two or three deep. From the rings small centrifugal branches pass between the bases of the surrounding spines of lower orders.

In addition to the above, there are a few iridophores scattered singly or in small groups over the interambulacrum, aboral to the area which bears the primary spines.

Below the ambitus the bands of iridophores follow a wavy course, becoming thinner and more compact, losing many of their branches. As they approach the peristome the bands break up into progressively smaller sections, separated by increasing gaps, finally disappearing before the peristome is reached. Because skin pigment is less dense here, many of the bands of iridophores can be seen to overlie the sutures between the test plates.

There are few iridophores in the ambulacra, where they are scattered irregularly between the bases of the spines.

THE COLOUR PRODUCED BY IRIDOPHORES AND ITS RELATION TO SKIN PIGMENT

When examined in their natural position by light reflected from a tungsten lamp, the iridophores appear predominantly blue, lilac, or turquoise. Each iridophore may be uniformly coloured or show patterns of the three colours in which blue and lilac predominate. The intensity of light reflected is not uniform, but areas of greater brilliance appear as fine lines which may be isolated or grouped in parallel or concentric array, and disposed with respect to the surface in many different ways. When the direction of the incident light is altered, the pattern of both the coloured areas and the lines of high intensity may change completely or disappear, but the range of colour does not alter.

The coloured light thus reflected produces a bluish glow in the surrounding areas of the skin, which is particularly evident in the grooves between the spine bases and alongside the white pattern developed in the interambulacra and around the periproct.

The quality of the colour is profoundly affected by the background provided by the skin pigment. This changes markedly in the vicinity of many iridophores, owing to the activity of the surrounding chromatophores (both black and red), which are affected by the prevailing light intensity. When the pigment is fully concentrated the iridophores overlie a white background due to the test, appearing almost colourless and difficult to see (fig. 1, B). In bright light the chromatophore pigment disperses so as to obliterate the white pattern. As the tongues of pigment encroach on the white areas, the outlines of the iridophores and their blueness become increasingly evident (fig. 1, C, D). At first they are predominantly lilac, but as more black melanophore pigment comes to lie beneath them they change to blue (fig. 1, D). This accounts for the lilac and blue pattern mentioned above. As the black pigment continues to disperse, the iridophores come to lie on a background that is completely black and they then reflect only blue.

The importance of the background effect is shown directly by lifting the blue iridophores away from the underlying melanin by microdissection needles and deflecting them on to a paler part of the skin, when their colour reverts to lilac, perhaps mixed with bluish green. When excised, placed over matt black paper, and viewed by reflected light, their blueness again increases although not to the same degree as when observed in their natural position; considerable admixture with lilac and blue-green persists.

It will now be evident that the iridophores play a significant role in the complex play of colours that accompanies the dispersion of each pigment in the skin, and the effect is partly due to their changing background. This change was described as seen in young individuals, in which it is most extensive (Millott, 1952). As urchins age, the progressive accumulation of pigment, most of which is static in the sense that it is not subject to rapid dispersion and concentration, lessens and in some cases abolishes the change. Other factors, too, such as changes in the chromatophores, may be involved. However, it is most significant that the capacity for pigment movement is retained longest in the melanophores which border the gutter-like depressions of the interambulacra. It is these which lie below the greatest concentrations of iridophores. But even here there is differentiation, for where the iridophores lie along one side of the gutter, the melanophores beneath them are more numerous and more active than those on the other side (fig. 1, B, C). Further, their pigment tends to remain dispersed for a longer time and few become punctate.

THE STRUCTURE OF LIVING IRIDOPHORES

When excised and examined in sea-water, the iridophores appear as more or less globular transparent bodies. They are usually lifted off with their underlying cushion of melanin and naphthaquinone, some of which is present in the amoebocytes which swarm around them. Varying numbers may be associated in short strands (fig. 1, E).

The iridophores may lie flush with the epidermal surface as in young individuals, or they may stand out, forming a prominent ridge.

Each iridophore is surrounded by a tough capsule (fig. 2). Its contents, which appear faintly yellow by transmitted light, show a striated appearance, suggesting a laminated structure. Sometimes the striations can be seen to form definite patterns or systems, which are complicated and variable. Some extend across the capsule as groups of parallel and more or less straight lines. Very commonly they are folded in a simple way, so that the parallel array resembles a nest of letters 'U' of graded size fitting closely into one another. Others form concentric systems, scrolls, or formations too varied to describe.

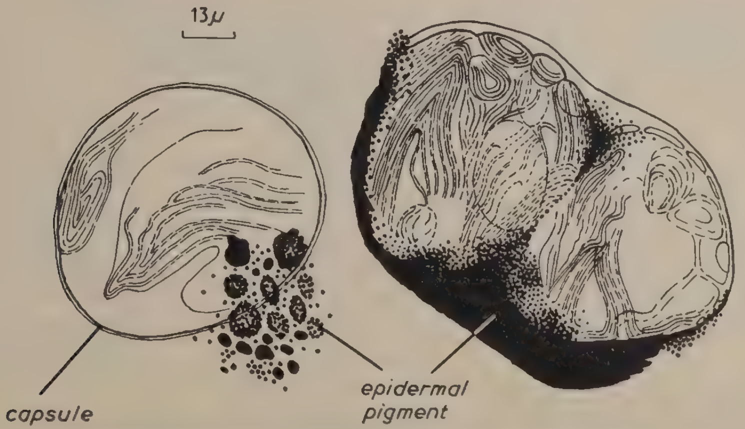

FIG. 2. Excised living iridophores, redrawn from preparations in sea-water (see p. 185).

Where they are clearly visible, the arrangement of the striations suggests that they are paired, each member of the pair being separated by about 1 μ, with a distance of about 2 μ between the pairs. Though they are much more numerous, their disposition recalls that of the bright lines observed by reflected light (p. 183).

Microdissection reveals more of the contents. If an iridophore is cut open, the contents protrude as a laminated, gelatinous mass, one end of which remains firmly anchored to the inside of the capsule.

When the capsule is punctured, some of the contained jelly escapes, leaving well-defined channels in the remainder. Squeezing such iridophores expresses the contained gel as separate masses showing a laminated structure, or as hyaline globules which pass along defined channels to escape by the puncture, leaving behind the walls of the channels as an array of parallel striae arranged along the lines of flow by which the jelly escaped. In form and arrangement the striae recall some of those just described in the intact iridophore.

The expressed gel shows remarkable properties. When compressed, it does not disperse into sea-water but spreads out to form a stratified mass of regular laminae, which appears to change or 'set' (fig. 3). The laminae gradually separate, forming rods of jelly that eventually break up into pieces of varying length and about 1 μ thick.

A most significant property is their bright blue appearance when viewed by reflected light over a background of black paper, which was evident whether the contents escaped in a single mass or as separate globules. In the former case, the gel showed the brilliant striations characteristic of the intact iridophore, and the degree of colour achieved was at least as great as when the contents were in the natural position.

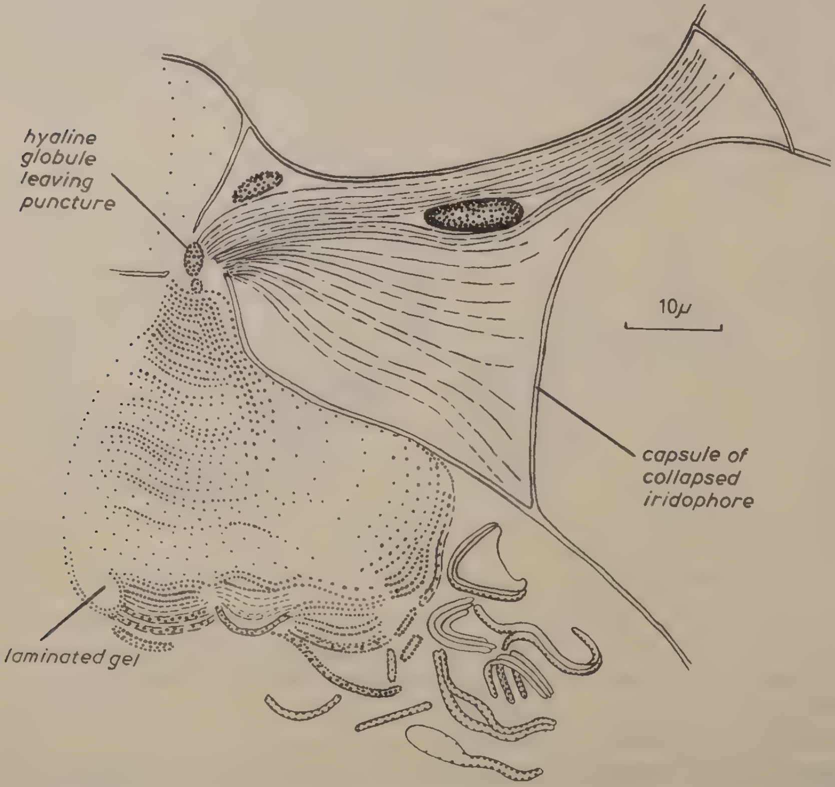
hyaline globule leaving puncture
10μ
capsule of collapsed iridophore
laminated gel

FIG. 3. Expulsion of the contents of a living iridophore into sea-water (see p. 185). Redrawn from a preparation subjected to pressure.

There is thus no evidence whatever for the existence of the solid plates or crystals that are usually associated with iridophores. On the contrary, the foregoing evidence from manipulation of living iridophores consistently indicates that a large measure of their contents is formed into regularly arranged gelatinous laminae, the disposition of which in some degree resembles the systems of lines visible in the intact iridophore. The peculiar layering properties of the gel might be related to its laminated natural disposition, and it is worth recalling that the layers tend to separate with about the same thickness as the distance separating the paired lines which divide up the contents of intact iridophores. This property also hints at an orientated ultrastructure, and the blue colour of the gel suggests the occurrence of light-scattering by colloids with a fine dispersed phase.

THE EFFECT OF FIXATION ON COLOUR

Elucidation of more detailed structure necessitates the study of fixed material. If any inference is to be made from such studies concerning the origin of colour in the iridophores, the effect of fixation on their colour should be known. Mortensen (1910) reported that the blue colour was not preserved by alcohol or formalin, and this has been found to be true for most of the fixatives we have used. However, we cannot assume that this is entirely due to the effect of fixation on the iridophores themselves, for some fixatives burst the chromatophores (Millott, 1953*a*), causing them to discharge their contents over the surface, and this might make the iridophores invisible without destroying their intrinsic colour-producing mechanism.

Pieces of test-bearing skin were fixed in formaldehyde-saline, Bouin, a modified Bouin (Atkins, 1937), Duboscq–Brasil, Carnoy, Heidenhain's 'Susa', Flemming's strong fluid, and Champy. Only the last preserved any of the blueness, which survived the subsequent washing in water, dehydration in ethanol, and clearing in methyl benzoate.

To discover whether fixation had transient effects on the production of colour, which might be significant, the action of several fixatives was followed under a microscope by delivering a few drops on to a cushion of iridophores viewed by reflected light.

Champy destroyed the lilac colour, so that the iridophores reflected a paler but more uniform blue, though the parts immediately overlying the melanophores still appeared more blue than the rest. No discharge of skin pigment was observed, and the capsule became noticeably whitish and less transparent.

Flemming's fluid rapidly destroyed the intense blueness. Most iridophores became opaque white, while a few continued to reflect a very pale blue which disappeared on subsequent washing in tap-water. A unique and noteworthy effect was to cause a small number to reflect intense red.

Bouin, Susa, and Carnoy completely destroyed all blueness, the iridophores rapidly becoming white and opaque. The action of Carnoy was instantaneous, and that of Susa required 5 to 10 sec. Very shortly afterwards they liberated floods of pigment from the surrounding red chromatophores, which rapidly turned brown to form a sooty deposit over the surface obscuring the iridophores. Thus the destruction of colour occurs first, and is independent of pigment discharge.

It may be mentioned here that osmotic changes, produced by immersing living iridophores in mixtures of sea-water and M/4 KCl or in distilled water, affect the colour. The former abolished it, but not permanently, for it returned on re-immersion in sea-water. Immersion in distilled water destroyed the blue colour, which partly returned in sea-water, but to what extent the effect on the iridophores is reversible remains unknown because much pigment is discharged.

THE STRUCTURE OF FIXED IRIDOPHORES

Paraffin sections 4 to 6 μ thick were prepared of skin and test. Owing to the kindness of Professor D. M. Steven of the University College of the West Indies, we were able to extend this part of our study to include iridophores of young animals measuring about 0·6 cm across the ambitus. Many staining techniques were used, including Delafield's haematoxylin and eosin, Masson's trichrome, Heidenhain's iron haematoxylin (alone and with light green), Weigert's haematoxylin (with light green or ponceau fuchsin). In addition, a number of special methods were employed, such as silver staining for reticulin (Wilder, 1935), alcian blue for acid mucopolysaccharide (Steedman, 1950), and the periodic acid / Schiff and Feulgen techniques.

In size and form, fixed iridophores approximate to the living, but it is possible to see that there is great variation in size among them even within one cushion, where the smallest are about 20 μ in diameter and the largest over 5 times this width. Iridophores of young animals are much smaller, being commonly about 20 to 30 μ across.

It is now possible to see that despite their superficial position they lie below the epidermis, which is reflected over them, changing its character to become a shallow layer of more or less cuboid cells (compare fig. 1, G), recalling the so-called 'cornea' of the Sarasins' description. Nothing corresponding to the cuticle they described in *Diadema setosum* exists here, though the epidermis is usually covered by a thin, even layer of apparently mucoid secretion, which stains brightly with alcian blue.

The capsule (fig. 1, G) lies immediately beneath the epidermis and is largely an elaborate network of branched fibres apparently continuous with those of the epidermal basement membrane, which they resemble in form, arrangement, and staining properties. Sometimes the capsule may be drawn out into a stalk of varying length (*st*, figs. 1, H; 4), anchored to the deeper layers of the test by a bunch of prominent fibres recalling those figured by the Sarasins. The fibres appear brown with silver and stain sharply with the periodic acid / Schiff technique; some respond to alcian blue. Interspersed among the fibres are small cells which form a more or less regular layer (*cap*, fig. 1, G) over the top of the iridophore beneath the shallow epidermis. Round the sides and beneath, the cells become sparse and attenuated so that, apart from their compressed nuclei, it is difficult to discern their presence. Where

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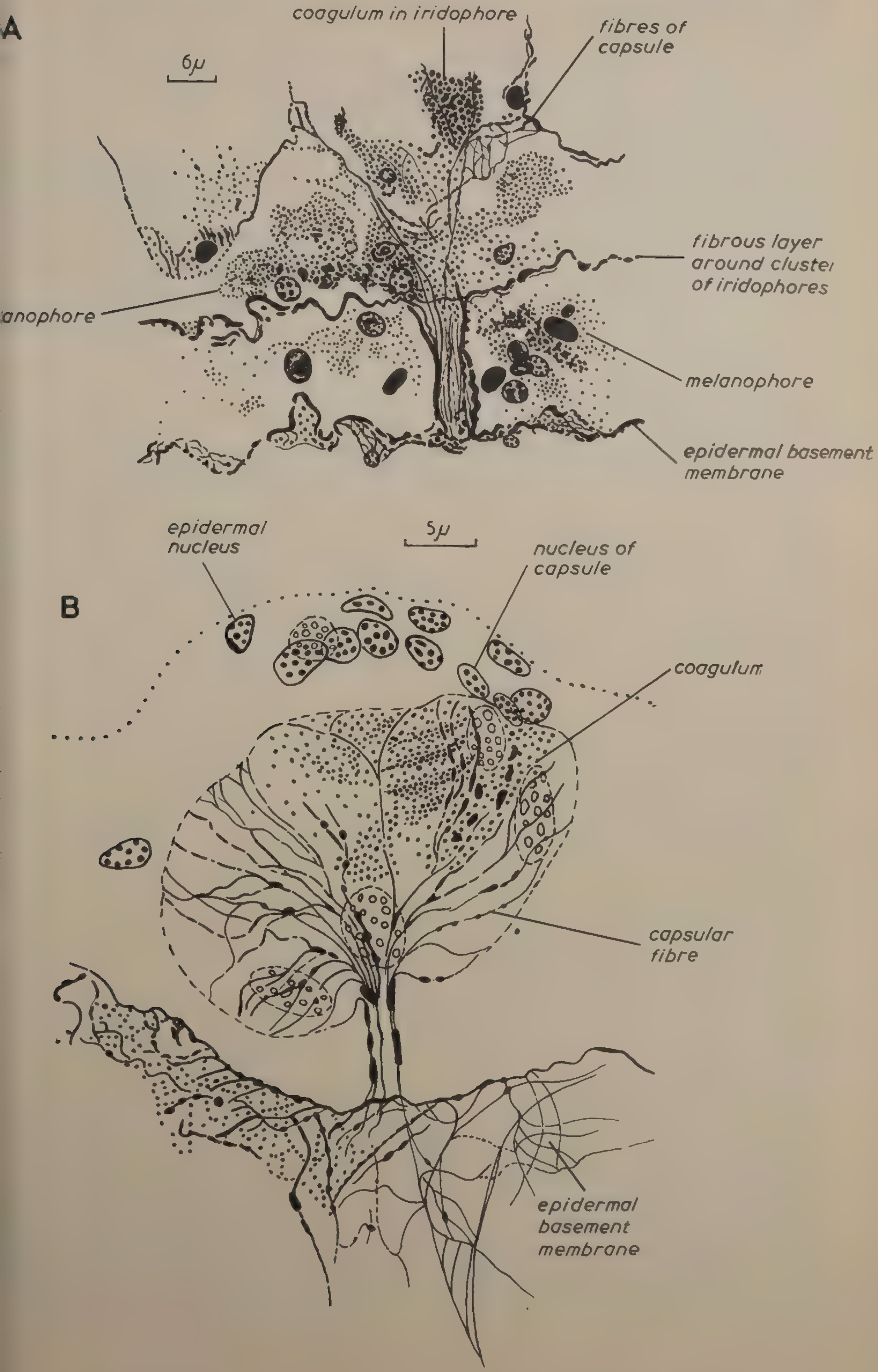
FIG. 4. Details of the capsule from preparations stained to show the fibres. A, basal portion of two iridophores, showing the relation between the stalk (of the iridophore on the right), the common fibrous layer extending around the cluster of iridophores, and the epidermal basement membrane. Fixed in formaldehyde-saline; silvered for reticulin. B, portion of the capsule of a single iridophore of a very young individual. The stalk and some of the capsule lie along the plane of section; the main body of the iridophore, being obliquely disposed, is below this. Some of the nuclei of the capsule, with some of its contents in the form of a coagulum, appear in deep focus. Fixed in Bouin: silvered for reticulin.


A
coagulum in iridophore
fibres of capsule
6μ
fibrous layer around cluste
of iridophores
anophore
melanophore
epidermal basement membrane
epidermal nucleus
5μ
nucleus of capsule
B
coagulum
capsular fibre
epidermal basement membrane

iridophores are compacted into groups, the whole is bound together by an additional fibrous layer (fig. 4, A).

The contents appear in the same bewildering variety as in the living structures, but it is now possible to see that they are a variable mixture of plates, coagulum, and what appear to be fibrillae.

After fixation in Champy (which also preserved the colour) the capsule contents took the form most closely resembling that seen in life, appearing as regularly arranged lines about 0·3 μ thick, grouped in pairs (fig. 1, G, H), each pair being separated by spaces about 1 μ wide and folded to varying degrees so as to form the same variety of patterns. Correlation of sections in different planes confirms what was already suspected (p. 186), that the patterns result from systems of variously folded partitions inserted by their edges on to the capsule (fig. 1, H). Those folded like a letter 'U' are most commonly inserted by their edges on to the region of the capsule which lies immediately below the epidermis.

The double lines form the thickened margins of plates (fig. 1, H). Each plate consists of a hyaline central core about 0·3 μ across, staining feebly with the periodic acid / Schiff stain and not at all with alcian blue, invested by a sheath of similar thickness which is coloured brown by osmium tetroxide, red by periodic acid / Schiff; it takes up light green but not alcian blue. The sheaths, which form the patterns of lines, are continuous with the capsule. No substance is visible between such plates. But not all the plates are like this even in the same iridophore. Some may appear swollen and distorted, with their sheaths separated from the core. Others may appear incomplete, failing to join the capsule, with gaps in the core and sheaths, either one or the other of which may be partly or completely lacking. The spaces between such distorted plates is usually occupied by a coagulum.

Other methods yield different appearances. After Carnoy or Susa fixation, particularly in conjunction with the periodic acid / Schiff technique (fig. 1, F), the plates are thin, often lacking sheaths and showing signs of dissolution, but they retain their regular arrangement so that the contents look rather like a thumb-print. Strongly PAS-positive droplets appear between the plates. Elsewhere the plates may be bundled together and often their identity is lost, the capsule being filled with coagulum which may be more or less homogeneous or pervaded by PAS-positive droplets or by what appears to be a tangled mass of fibrils. Shrinkage and distortion of the plates is particularly marked after fixation in Bouin.

Sections in different planes show that the fibrils are fragments of the sheaths. Further, there is a roughly reciprocal relationship between the number, size, or integrity of the plates and the amount of coagulum or the number of PAS-positive droplets. This suggests that the varied appearances represent stages in the progressive distortion and eventual breakdown of the sheathed plates under the action of fixatives, the material derived from disintegration accumulating between the remains of the plates as coagulum or droplets. The idea is confirmed by appearances after the PAS technique when the sheaths of the plates stain progressively less as the amount of red-staining material between them increases.

It is clear that fixed material has strictly limited usefulness and can only be interpreted in conjunction with the living structure.

The notion of sheathed gelatinous plates would fit in with the behaviour of living iridophores under compression already described (p. 185). Thus if the hyaline core alone were expressed (and the sheaths are now known to be anchored to the inside of the capsule), not only would it tend to escape along definite channels walled-in by the sheaths, but the empty sheaths left behind would appear as the parallel striae observed. Moreover, their arrangement would be expected to resemble that of the striations seen in living intact iridophores (p. 185).

THE PRODUCTION OF COLOUR

The means whereby the iridophores produce their blue colour presents a difficult problem. The blueness of the iridophore contents (p. 186) shows that it is not due in significant measure to the superjacent tissue as previously conjectured (Millott, 1953 *a*, *b*, *c*).

The fact that the blue is obvious when the iridophores are seen by reflected light, yet is lacking when they are viewed by transmitted light, indicates that the colour is produced by structural means rather than by pigmentation. Possible participation of pigment is not ruled out by this, however, because there might be minute amounts of blue pigment in the iridophores, insufficient to be evident when light is passed directly through them but sufficient to produce obvious colour when disposed in a thick layer. The equivalent of such a layer might be produced if light were passed by internal reflexion along the laminae, acting as light guides in such a way that the incident light passed down one limb of the 'U' to be reflected back along the other. The yellowish tinge observed when iridophores are viewed by transmitted light might be attributed to the naphthaquinone which often surrounds their bases.

However, the production of blue by pigment means that significant absorption of spectral yellow must occur, so that the possible participation of pigment can be tested by examining iridophores by reflected yellow light. If such absorption takes place, the iridophores should appear dark. They do not. When viewed by light passed through a Wratten No. 12 filter (which transmits maximally between 550 mμ and 700 mμ and cuts off all visible light of wavelength shorter than 500 mμ), they appear yellow. There is thus no significant absorption of yellow light, and therefore no evidence of blue pigment.

The use of an Ilford filter No. 805, to remove any ultra-violet in the light beam by which iridophores are examined, makes no appreciable difference to their blue colour. Again, iridophores are invisible in ultra-violet light, so that fluorescence plays no significant role.

The evidence thus supports the previous indication that the colour is structural in origin. It may result from refraction, interference, diffraction, or scattering.

The first is eliminated, because when the iridophores are viewed in the natural position against a black background the only colour visible is blue, and there is no change when the angle or direction of the incident beam is altered.

Interference and diffraction are more difficult to eliminate, and it must be admitted that the structure of the iridophores with their regularly arranged plates hints at such an origin for their colour. The spacing of the plates is necessarily a critical factor in production of colour by these means, but we have not been able to obtain measurements that are sufficiently reliable to test the idea. On the other hand, stretching and compressing iridophores by microdissection would be expected to alter the spacing of the plates, in view of the gelatinous nature of the iridophores, but it does not alter the colour of the light reflected from them. Again, the intense blueness evident in drops of gel expressed from iridophores suggests that critical spacing of the plates is not a major factor, although it is impossible to be certain that such droplets did not contain intact fragments of the laminated structure. Further, there is no directional effect, blue being reflected in all directions, and the colour does not vary appreciably when the incident beam is rotated through an angle of 35° (the maximum possible with the optical means available). Though this evidence is not conclusive, because the systems of the plates within one iridophore may be disposed at a variety of angles with respect to the surface, movement through such an angle would be expected to make an appreciable difference to the colour; but this was not observed.

Scattering seems a more likely source of the colour. The obvious preponderance of blue in the emergent beam, as well as its intensity, would be expected with fourth-power scattering. The slightly yellowish colour shown by iridophores when light is passed through them and the purification of the blue colour which occurs when iridophores are viewed against a black background agree with this idea. Again, when iridophores are examined by polaroid filters the beam reflected from them is seen to be polarized. In the balance, therefore, such facts favour scattering as the means of producing colour.

The intensity of blueness observed in structures so small as the iridophores would suggest Rayleigh scattering by an optically heterogeneous system with particles less than 0·1 μ in diameter, and the delicate colloidal plates of the iridophores could embody such a system.

DISCUSSION

To what degree the iridophores of *D. antillarum* correspond with the blue spots in other diadematids is uncertain. Nevertheless, in their position in the skin, as well as in the general features of their distribution and structure, the iridophores resemble the blue spots described by the Sarasins in a species from Ceylon which they called *D. setosum* Gray (see p. 181). In each case the structures are seated among pigment cells and rest on the superficial nerve-layer, being bounded externally by the epithelium covering the test, which is here greatly thinned out.

Caution is necessary in attempting detailed comparisons, because existing descriptions of the structures in *D. setosum* are based on fixed material alone. Again, doubt has been cast on the accuracy of the Sarasins' account (Cuénot, 1891; Mortensen, 1940), parts of which the authors admit can be accepted only with reservation because it applies to structures that they found difficult to interpret and which changed considerably on fixation. Neither this, nor lack of experimental evidence, appears to have influenced their conviction that the blue spots were eyes!

Nevertheless, certain features they described can be matched with those of the iridophores of *D. antillarum*. Thus their 'cornea' corresponds with the thin epithelium overlying the iridophore, while their 'nuclear cap' seems to correspond with the nuclei of the capsule cells lying beneath the epithelium. It is difficult to match any structures with the so-called 'retinula'.

The contents of the capsule seem to have proved something of an enigma. The Sarasins interpreted them as lenses formed of more or less regularly arranged, vacuolated cells, while Cuénot (1948) seemed inclined to regard them as mucoid. Mortensen (1940, p. 248) is curiously inconsistent. Accepting the view that the whole structure is an eye, he disagrees with the Sarasins' figures and describes the contents as a 'dense mass of fibrillae wound up like a ball', yet he figures them as what might be a uniform coagulum (fig. 12, plate LXXIII).

Examination of the very few specimens of *D. setosum* to which we have had access reveals that the contents not only show signs of a laminated structure but also a variety of appearances which parallel those seen in the iridophores of *D. antillarum* after fixation. This not only strengthens the previous conjecture (Millott, 1953*a*) that the blue spots in the two species are produced by the same kind of structure, but also suggests that changes which follow fixation of the so-called 'eyes' of *D. setosum* may have been partly responsible for the different and confusing descriptions just mentioned. Re-investigation of these structures is long overdue.

We are deeply indebted for assistance to the Zoological Society of London, particularly to Dr. H. G. Vevers. It is also a pleasure to acknowledge the help and advice we have received from Dr. E. J. Bowen, F.R.S., and Dr. J. W. Smith, the gifts of specimens from Professor D. Steven and Professor Tomiyama, and the assistance in photomicrography from Mr. Maurice Gross.

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The Structure and Function of the Cutaneous Glands in *Helix aspersa*

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SUMMARY

The glands discharging 'slime' on to the surface of the mantle collar and foot of *Helix aspersa* have been investigated histologically and histochemically on chemically fixed and frozen-dried material.

All the glands are unicellular; they lie in the connective tissue and discharge by pores passing between the epidermal cells; some are club-shaped, others are polygonal with a distinct and usually long duct. At least 8 different kinds of gland are found: 4 extruding various kinds of mucus, one protein, one calcium carbonate granules, one a pigmented secretion containing a flavone, and one releasing fat-globules. The histology of the mantle collar is very similar to that of the dorsal and lateral surfaces of the foot except that the glands in the mantle are usually larger.

All kinds of secretion are extruded from these parts. The glands of the sole of the foot are mostly of a distinct kind and produce mucus combined with protein.

The mechanism of discharge is discussed: some of the gland cells are enclosed in a network of muscle-fibres which are thought to be concerned in the removal of the secretion, in other cases no fibres have been found and it seems likely that changes in pressure in the haemocoel are involved.

The composition of the slime changes from colourless and viscous to yellow and watery when the animal is irritated. It is usually slightly alkaline, is not distasteful to man, and does not inhibit the growth of micro-organisms. The mucous component acts as a lubricant, and on the sole for adhesion. The calcium carbonate granules and the protein may be concerned in defence, while the flavone is a waste-product varying with the amount of green food eaten.

The epiphragm is formed by the secretion of part of the mantle collar and is probably dissolved away by a protein-splitting enzyme which has been demonstrated in the slime.

INTRODUCTION AND METHODS

GLANDS are present either in or below the epithelium of most external surfaces of molluscs. Beneath the shell and in the mantle groove lie cells which are concerned with shell formation, described by various workers in the past; this paper is concerned with those glands discharging on to outer surfaces which are not covered by the shell, and no mention will be made of either the pedal or the hypobranchial glands since they too have been previously considered.

The slimy covering of a mollusc is frequently referred to as 'mucus', a term with no precise meaning. Observation of a living specimen of *Helix*, however, suggests that the slime is not always of identical composition, and the histology of the structures concerned reveals that a variety of secretions is in fact produced.

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Some detailed investigations have been made in the past: de Villepoix (1892), Prenant (1924), Heaysman (1951), and Russell (1954) all described *H. pomatia*, and other authors have worked on other genera. However, apart from that by Russell, most of these descriptions are of the histology of the glands and the appearance of the secretions, with little detail of the physical and chemical nature of the products. This investigation, although involving cell structure, has concentrated on the histochemistry of the glandular exudates. The attempt was made to determine the function of each kind, an aspect which has previously received little attention.

For histochemical work the choice of fixative is important. It is desirable to avoid mordants such as mercury, aluminium, or chromium, to avoid an acid medium in order to retain calcium salts, and to use formalin which is reputed to fix proteins without rendering them inert. The fixative most frequently used was that of Glick (1949), a 1:1 mixture of 15% formalin and 8% basic lead acetate, fixation being carried out for about 16 h. A white precipitate forms around the material with this mixture but usually this can be adequately removed by shaking or by wiping with a paint brush. Although this method gave reasonably good histochemical results it was unsatisfactory for critical histological work, being far too drastic in its shrinkage effects. For this part of the investigation either Susa or Flemming-without-acetic was used. By far the best results from the point of view of fixation were obtained by using an Edwards's freeze-drier. By this method contact with chemicals is avoided until the sections are dewaxed, passed through the alcohols where fixation occurs, and hydrated before staining. Shrinkage and distortion are minimized (with the exception of one kind of mucus), and the protoplasm retains a much finer texture. There are, however, certain drawbacks. Serial sections cannot be guaranteed, blocks of tissue must not be larger than cubes of side 3 mm, and orientation of the blocks is therefore difficult. The histochemical tests revealed other intricacies which will be discussed later.

Large numbers of tests were carried out initially, including those to identify mucopolysaccharides, protein, fat, calcium, iron (both ferrous and ferric), strontium, copper, zinc, and phosphorus. For mucopolysaccharides aqueous toluidine blue (about 0·1%) was found to give the best results. Staining time increased from 5 to 10 min with the age of the solution, which, however, gave good coloration for several months; this resulted in over-staining, differentiation occurring during dehydration. The material was inspected in distilled water immediately after staining because the metachromatic effect decreases in contact with alcohol, and a substance staining an intense pink may appear blue after dehydration. However, this reversal of metachromasia proved useful in comparative work since of two secretions both appearing pink in distilled water one might return to blue and the other retain a considerable amount of its pinkness after dehydration. Therefore, immersion in alcohol proved a useful way of differentiating between different kinds of mucus. All the results described here are those obtained after dehydration and the making of permanent mounts. It was found that viewing the sections in artificial light often enhanced the metachromatic effect. Alcian blue as a second stain for mucopolysaccharides was utilized as described by Steedman (1950), except that for more precise results a longer time of immersion in a much weaker solution was preferable since it completely avoided background staining. Occasionally the PAS technique was employed, according to the directions of Pearse (1954).

Heidenhain's iron haematoxylin gave the best demonstration of the product from the protein glands; Millon's reagent and ninhydrin stained positively, although rather evanescently, and only when large amounts of secretion were present.

Calcium could be displayed most readily by silver deposition by the von Kossa method (Gomori, 1952). Although the precise strength of the solution is not important, 5% for 5 min gives good results. An alkaline fixative should be used, although Russell (1954) considered one of pH 4·7 suitable. Alizarin red S or nuclear fast red also proved useful. Russell, working on *H. pomatia*, employed very long staining times with both von Kossa and nuclear fast red; these should be avoided, since not only is it unnecessary but it leads to darkening or colouring of the background. Control sections were treated with 1 N HCl for 5 min before immersion in the staining solution.

Pearse's molybdate test for phosphate, and Sudan black for lipids were also used.

For general histological investigation alcian blue was used in conjunction with Heidenhain's haematoxylin and eosin; the more dilute the alcian blue the clearer and more distinct the result, the best results being obtained by immersion for 12 h in a scarcely coloured aqueous alcian blue after differentiation of the haematoxylin, followed by dehydration, with a little eosin in the first absolute alcohol. This gave a bluish-green colour to the mucus, and this contrasted well with the normal haematoxylin / eosin combination.

This paper is concerned with *H. aspersa*, a species not well described by earlier workers, but similar enough to *H. pomatia* for the latter to be used as a basis for comparison. Most, if not all, of the glands in the skin of gastropods are unicellular; each gland cell will therefore be referred to as a gland. In accordance with the terminology of Bowen (1929), 'secretion' will be restricted to the formation of the product from the cytoplasm within the cell, while 'extrusion' will be used for the removal of the product from the cell.

RESULTS

Although some glands are constant in appearance, considerable variation occurs among others. It is reasonable to assume that the physical and chemical nature of the secretion may change during its formation within the cell, especially as some of the substances involved may be excretory and vary with the conditions under which the animal had been living. The size and probably also the shape of the cell will vary according to its age and also perhaps with the stage of its secretion; after extrusion of the contents a decrease in volume would be expected. It is therefore perhaps not surprising that a certain amount of difficulty has been experienced in deciding how many distinct kinds of glands are found in *Helix*. However, taking all these factors into consideration, 8 types of gland cell, including 4 producing different sorts of mucus, have been distinguished; for reference purposes these have been called:

mucus gland type A
mucus gland type B
mucus gland type C
mucus gland type D
protein gland
calcium gland
pigment gland
lipid gland

Three regions have been investigated, namely, the mantle-collar, the dorsal and lateral surfaces of the foot, and the sole of the foot. The glands in the mantle region of *Helix* are larger than those elsewhere and will be described first. Those on the dorsal and lateral surfaces of the foot resemble those of the mantle in many ways and will be considered later. Finally, the glands of the sole will be discussed.

The mantle-collar

This may be subdivided into two regions: the larger part adjoining the shell gland (the ventral surface), and the other lying approximately at right angles to it and adjacent to the sides of the body (the median surface).

The ventral surface of the mantle. The gross appearance of the collar varies from specimen to specimen, but it is always more or less glistening owing to the presence of its covering of slime. Under this it may be dark grey or almost cream, invariably mottled, and with the region around the pneumostome whiter than elsewhere. This surface of the mantle is covered by a single layer of columnar cells from 25 to 33 μ deep, bounded by a distinct cuticle. The presence or absence of cilia has received much comment in the past. Cilia are always to be found around the pneumostome, but elsewhere they occur only on small scattered groups of cells, and not continuously as described by Heaysman (1951). Beneath the epidermis lies the loose connective tissue in which the glands are found. All the gland cells are similar in consisting of a more or less swollen basal part opening by a narrow and well-differentiated duct or by a broad neck between the epidermal cells. Except in young stages very little cytoplasm can be seen.

The general shape of the mucus-glands of type A can be seen from fig. 1, their length being up to 800 μ. A nucleus is visible only in the smaller cells where it lies basally, becoming more and more compressed in larger glands until eventually it is invisible. The secretion is not granular, frequently appearing reticular or 'bubbly'. After freezing-drying the mass of mucus often seems to have 'exploded', leaving trails of the material dispersed over the surrounding tissue; occasionally this happens after chemical fixation, and is probably due to the sudden effect of the fixing agent. The secretion stains with alcian blue, neutral red, Mayer's mucicarmine, and (faintly) with PAS. It shows gamma metachromasia with toluidine blue, Terry's polychrome blue, and thionin.

The distinguishing characteristic of the mucus-glands of type B is the clearly and evenly granular nature of their secretion. The cell-body is polygonal and comparatively small, opening by a fine duct which passes through the connective tissue and between the epidermal cells, though its position can be seen only when granules are present as the walls are too thin to be visible alone. The nucleus remains spherical and centrally situated. The whole cell including the duct may be 300 μ long. The staining reactions of the secretion are similar to those for type A glands except that it is strongly positive with the PAS technique.

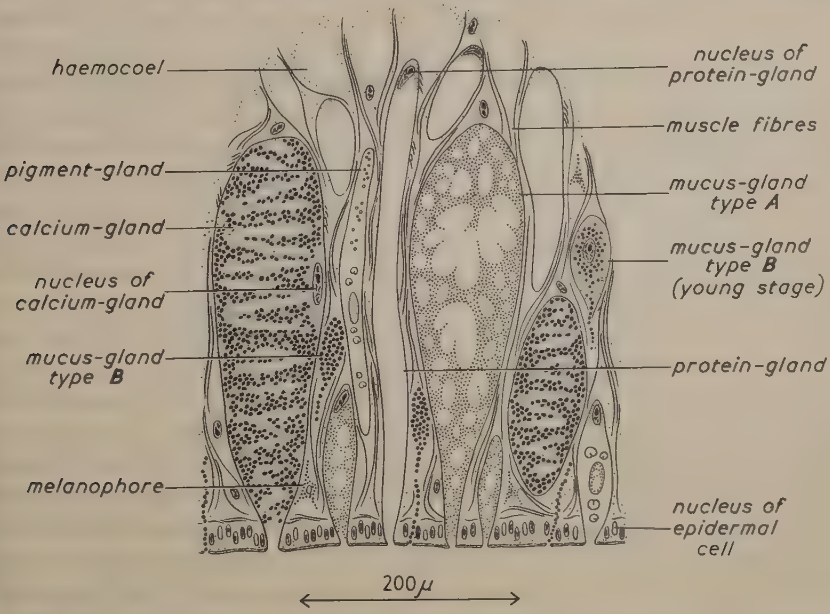

FIG. 1. Transverse section through the ventral surface of the mantle-collar of *H. aspersa*, after chemical fixation.

Previous workers have given various accounts of mucus-glands. Barfurth (1885) referred vaguely to mucus-glands, while Prenant (1924), using haemalum as his only stain for mucus, described two types; he did not find any protein glands and it seems more likely that he missed the second kind of mucus-gland and wrongly identified the protein. Roth (1929) distinguished, but not very clearly, two kinds of mucus-gland. Passing comment was made in 1951 by Heaysman about mucus-glands—presumably of only one kind; she alleged, however, that the secretion was found in the haemocoel and not in cells—although the associated nuclei can frequently be seen quite clearly. The most recent work (Russell, 1954) referred to one type of mucus-gland, though something which could well have been the type B cells was described but not identified either as mucus or as a gland.

The calcium-glands are the cause of the whitish mottling of the mantle and it is because they are particularly abundant around the pneumostome that that region appears whiter than the other parts. The cells themselves are similar in shape to the large mucus-glands, although rarely exceeding 550 μ in length. The secretion consists of granules which are either spherical or very short rods, suspended in a faintly yellow matrix of basic protein. The granules frequently obscure the nucleus. When it is visible, however, it is often found along the side of the cell, although it may be terminal.

Positive results with von Kossa, alizarin, nuclear fast red, and Edward Gurr's gallamine blue (all of which were prevented by previous immersion of the sections in 1 N HCl for 5 min) indicate that the granules consist of a calcium salt; the fact that they effervesce readily with acids indicates a carbonate. The ammonium molybdate test for inorganic phosphate gives variable results with this material. The colour is evanescent and rather pale but in some sections a blue halo around the calcium glands has been obtained and in others a certain blueness actually within cells which were probably of this kind. Calcium phosphate is somewhat water-soluble and therefore very little would be left after hydration of the sections, regardless of the method of fixation; however, tests involving as little contact as possible with water contrasted with others left in running water for 20 min showed no detectable difference. It therefore seems likely that the calcium is transported to the glands as phosphate but that it is stored as carbonate. The secretion is certainly extruded from the cells as carbonate granules since they can be seen in sections lying in the mouth of the gland and in the slime clinging to the outer surface. Heaysman's theory of transport through the epidermal cells and reconstitution on the surface of the mantle is therefore not supported.

Gray (1926), working on artificial membranes of mucin, found that dispersion was prevented by divalent cations. Further investigation on *Mytilus* gill tissue showed that the intercellular matrix, which is faintly positive with Millon's reagent, was stabilized by calcium and magnesium ions. It is possible that a similar thickening of the protein occurs in the calcium glands of *Helix*. After chemical fixation the granules frequently appear to be arranged in bands as indicated in fig. 1 (for clarity fewer granules are shown than are in fact present). This may well be due to shrinkage causing the protein matrix to contract or pull away in strips from the cell boundary.

Previous workers have all recorded calcium-glands.

Protein-glands appear abundantly in the collar. Since their length (up to 850 μ) tends to be greater than that of the other glands, their bases frequently appear to form a band at the inner edge of the glandular mass. Their shape is cylindrical, appearing somewhat ribbon-like in section, tapering only very little and opening by a wide pore between the epidermal cells. Their nuclei are larger than those of the other glands; they tend to be ovoid and lie in a more plentiful mass of cytoplasm at the inner end of the cell. The secretion is usually homogeneous, although occasionally very finely granular; positive reactions with ninhydrin and with Millon's reagent indicate that it is protein in nature. It stains with iron haematoxylin. This material in the fresh state has a distinct primrose colour; further comment on this pigment appears below.

In many specimens only these 4 types of gland are to be found in the mantle-collar; others, however, possess a fifth kind (labelled pigment-gland in fig. 1), and occasionally these occur in considerable numbers. In shape they are somewhat intermediate between the protein- and calcium-glands, though seldom as long as either. On rare occasions an ovoid nucleus is visible at the inner end of the gland, but their main characteristic is the deep yellow colour of the secretion.

Whereas in the other kinds of gland the secretion has been generally dispersed through the cell, either in single vacuoles or in granules distributed through the matrix, it exists in this type in various forms. Frequently a series of globules is present, of very variable size even within a single cell, each globule apparently with a distinct boundary since what appear to be cracks in it can sometimes be seen. Often one or more elongated structures are present which look very much as though they have been formed by the joining together end-to-end of several granules; the yellow pigment then seems to be concentrated on the surface and the inner part is paler. The rounding-off effect of these globules suggests an oily nature but no positive tests of any kind for lipid or any other substance have ever been obtained. The pigment probably masks any reaction to some extent. Although the pigment is evident in frozen-dried material before it is put into alcohol, none can be found later.

Pigment glands might perhaps be discharged protein-glands, in which case one would expect a positive reaction to be given at least by Heidenhain's haematoxylin. Roth (1929) and Russell (1954) both recorded pigment-glands, without giving much comment on their nature; other workers have made no mention of them.

Finally, cells containing globules of lipid giving a positive reaction with Sudan black must be recorded. They are rarely found and have been seen only in frozen-dried material. Lipid would be dissolved out during the usual chemical fixation, but none was detected after formaldehyde-calcium, which normally preserves fats. Although the shape of the cells might be due to pressure of the surrounding tissue it suggests that of a gland, especially as in some instances what would appear to be a duct leading towards the surface can be seen. No lipid has, however, been found actually in the discharged slime, and lipid has not been recorded in the skin of *Helix* before.

Occasional cells very similar to the type B mucus-glands but with non-metachromatic granules have been found; they appear to be like the type D glands to be described later.

The median surface of the mantle. The main difference between this surface, which lies adjacent to the foot and the ventral surface, is in the relative abundance of the various kinds of glands; otherwise it is similar, though with a general tendency for the gland cells to be smaller.

Numerous mucus-glands of the A type occur here; indeed, there may be a cluster of a dozen or more lying pressed closely together, but there is considerable individual variation. Mucus-glands of the smaller type are scattered and not so frequent. Both protein and pigment glands are much rarer than on the ventral surface; although calcium-glands may be found on the lower edge of this part, where it joins the major surface of the mantle, they are never found higher up.

This change in distribution may well be correlated with epiphragm formation. Russell (1954) stated that the mantle-glands deposit the epiphragm, but gave no further details. In this species it is largely proteinaceous in composition with a small amount of calcium. When the body of *Helix* is retracted into the shell, the foot contracts to such an extent that it is withdrawn completely through the collar. The inner edges of the ventral surfaces of the mantle are drawn together below the contracted foot, while the outer edges retain more or less their original position around the edges of the shell mouth. This means that it is the ventral surfaces of the collar which occupy the position just within the opening of the shell, and it is presumably from this part of the mantle that the epiphragm material is extruded. The presence of protein and calcium glands on the ventral surfaces and their scarcity on the lateral ones is probably linked with their use in the laying down of the epiphragm.

The dorsal and lateral surfaces of the foot

The general appearance of these parts of a snail's body is of a reticulum of grooves subdividing the surface into greyish polygonal areas. Microscopically, apart from the grooving, the structure is remarkably reminiscent of the mantle-collar, as may be seen from fig. 2, and the same kinds of glands are present, the main difference being in their smaller size, particularly on the dorsal aspect. There are isolated patches of ciliated cells in the epidermis, but the majority of the surface bears no trace of them.

Mucus-glands of type A occur commonly in this region, but there are fewer of type B; indeed, in some snails it is difficult to find examples. Occasionally intermediate forms are found, as well as specimens with metachromatic mucus. Protein-glands are less abundant than in the mantle, and this is also true, particularly on the dorsal surface, of calcium-glands; those containing either pigment or lipid occur sparsely as before. In each case the histochemistry of the secretions is identical with that in the mantle.

The sole of the foot

The sole of the foot is completely covered with fine cilia arising from elongated epidermal cells. This is the only region of the body, apart from the pneumostome, where cilia are invariably present. Beneath this layer lies a glandular zone extending to 450 μ deep. The general shape of the individual cells can be seen from fig. 3.

Glands of type C are very numerous in the sole, and often occur in clusters, although there is never any indication of syncytium-formation, as clear cell boundaries remain. The secretion in the cells occurs as distinct granules; that in the ducts, particularly near the surface, often appearing somewhat reticular. After chemical fixation it stains with alcian blue, faintly with PAS; with toluidine blue it shows gamma metachromasia. It is similar therefore to the mucus of the type A glands, but it differs in taking up Heidenhain's haematoxylin. The latter result suggests the presence of carboxyl groups, which, although probably in protein, might be in mucopolysaccharide. Tests with Millon's reagent and ninhydrin were, however, negative; but since these methods never give a strong coloration and since the granules are minute, this is perhaps not surprising. Mucopolysaccharides are frequently, if not always, associated with a small and varying amount of protein; indeed, Sherwin (1935), Hempelmann (1940), Ewer and Hanson (1945–6), and Pigman and Goepp (1948) have all considered mucoitin-sulphuric-acid and chondroitin-sulphuric-acid to be mucoproteins, although they should correctly, according to Meyer (1945), be termed mucopolysaccharides. One is therefore tempted to suggest that the secretion from these glands contains more protein than that from the types A and B mucus-glands. This would seem to be supported by the work of Suzuki (1940–1) and of Masamune, Yasuoka, Takahasi, and Asagi (1947), who investigated the chemical composition of mucus (presumably meaning slime) from the sole and other parts of the body of *H. laeda* and found two distinct types. Suzuki's figures show 13·9%

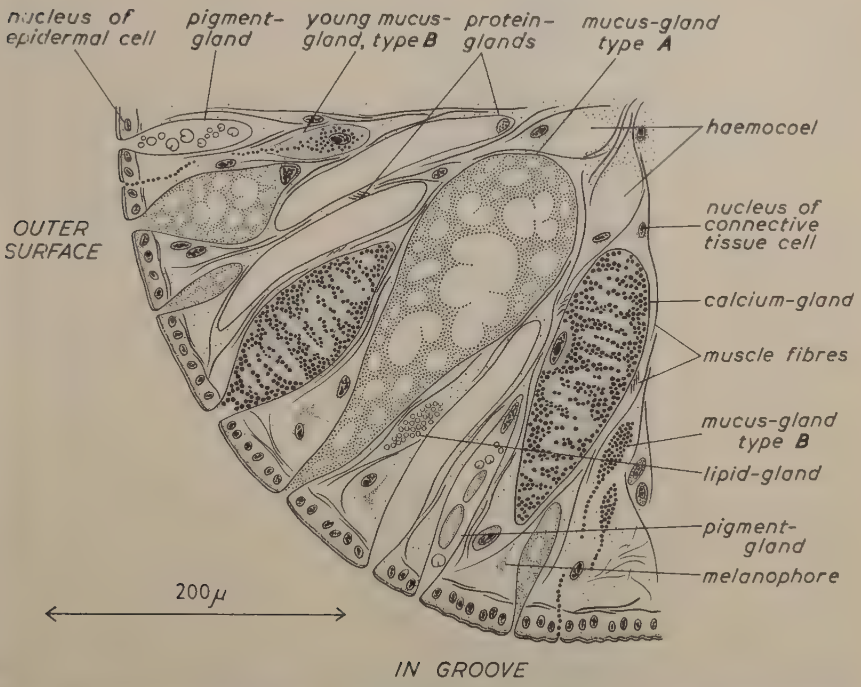

FIG. 2. Transverse section through the side of the foot of *H. aspersa*, after chemical fixation.

nitrogen in mucus from the foot, presumably the sole, compared with 12·9% for the rest. Assuming that the gland population of the species is similar to that in *H. aspersa*, the 12.9% would include exudate from the protein glands as well as mucus; this means that the 13·9% for the sole glands is very much higher than the percentage of nitrogen in the actual mucus-glands elsewhere.

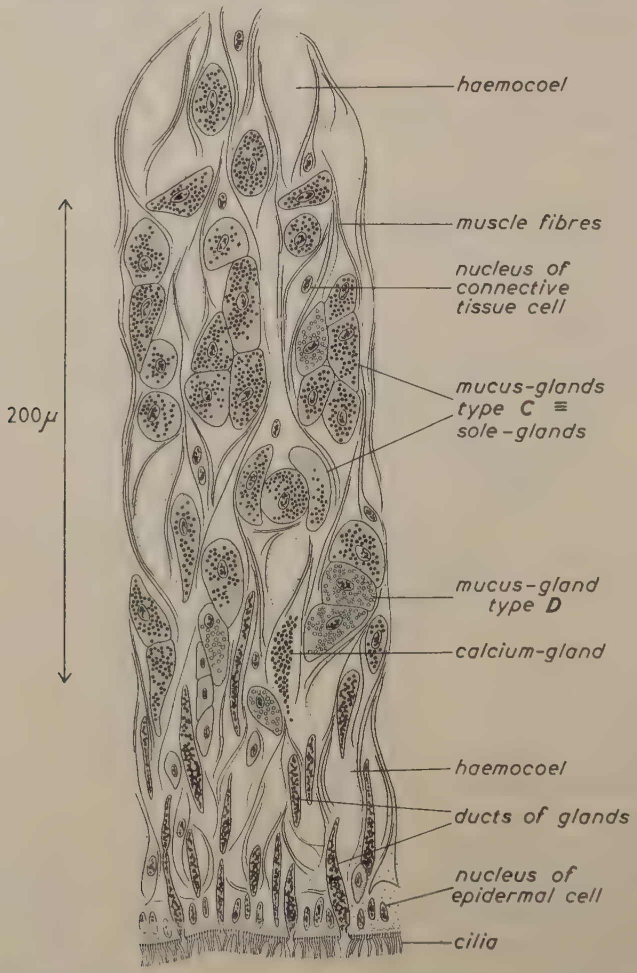
haemocoel
muscle fibres
nucleus of connective tissue cell
200μ
mucus-glands type C ≡ sole-glands
mucus-gland type D
calcium-gland
haemocoel
ducts of glands
nucleus of epidermal cell
cilia

FIG. 3. Transverse section through the sole of the foot of *H. aspersa*, after chemical fixation.

This work must, however, be treated with caution since the composition of the slime in *H. aspersa* varies with the strength of excitation—as shown later—and this could affect these results considerably.

According to Walton and Ricketts (1954) the binding of protein to mucopolysaccharides results in decreased metachromasia, in which case one would expect the sole glands to display less metachromasia than the A and B type glands, which is not the case. Certainly mucoproteins would not give gamma metachromasia, and one can only conclude that this secretion is a mucopolysaccharide, probably linked with some protein, and is distinct from that produced by the other mucus-glands.

Since these are the most numerous glands in the sole, they must be regarded as the ones which Roth (1929) and subsequent workers called sole-glands. However, Roth was wrong in saying that they also occur in the mantle: the secretion of the type B glands found there is quite distinct in histochemical properties.

Scattered among the sole-glands, particularly within 265 μ of the surface, lie occasional gland cells of type D. These appear very similar to those described above in general shape and in the granular nature of their secretion. However, on chemically fixed material the granules are positive to alcian blue and to PAS, but are not metachromatic with toluidine blue. In this they resemble the cells found infrequently in the other areas studied.

Some doubt exists as to whether the type D glands are a distinct kind. Cells can sometimes be found containing a very little secretion which has the type D characteristics; others are occasionally present which contain this material with some granules positive with Heidenhain's haematoxylin, from which it might seem that type D glands were simply early stages of type C. However, non-metachromatic material has sometimes been seen in ducts passing between the epidermal cells and it therefore seems likely that type D glands do discharge their contents, which means that they are unlikely to be young stages. It may be that both C and D mucus-glands have a common origin.

It is perhaps pertinent here to mention the development of the other kinds of gland. Opinion in the past has been divided into two schools of thought: that they are epidermal or that they are of connective tissue origin. Roth (1929) maintained that it is rare to see developmental stages of glands in mature specimens of *Helix* but that they occur in the unhatched egg and young animal. From this one is led to assume that some at least of the glands presumably function throughout the life of the animal. Certainly not many convincing developmental stages have been seen during this work, which has been mainly on snails more than one year old. However, periodically enlarged connective tissue-cells with obvious early stages of protein secretion or the various kinds of mucus secretion around the nucleus have been seen. Steps in the formation of a duct or elongation of the cell towards the surface have also been observed. It seems likely that at least some of the skin glands of *Helix* are of connective tissue origin.

Freezing-drying

The results of histochemical tests on the calcium secretion and various kinds of mucous secretions with frozen-dried material have been the same as after chemical fixation. The apparent absence of pigment glands has already been commented upon. However, the reactions of the protein secretions are completely different. Whereas after chemical fixation a positive result was obtained with Millon's reagent, ninhydrin, and iron haematoxylin, after freezing-drying no staining occurred.

This suggests that the protein is not bound during the process and is therefore washed away during hydration, or that it is bound so firmly that it cannot react with the stain (although this is unlikely), or that it is denatured in some other way. Formalin is reputed to bind protein, but immersion of the frozen-dried sections does not change the result. Little seems to be known of the effect of freezing-drying on protein. Proom and Hemmons (1949) showed that temperatures down to −78° C (that used here was −170° C) do not seem any worse than −17° C from the point of view of survival of bacteria. Haines (1938), working on freezing alone, showed that the critical temperature for bacterial survival, although varying with the species, appears to be about −2° C and that even after 8 days at this temperature only 50% of the coagulable protein had coagulated. An interesting point is that the secretion of the sole-glands, which was positive with Heidenhain's haematoxylin after chemical fixation, remains positive with it after freezing-drying. If the haematoxylin is demonstrating protein, this suggests that that bound to mucopolysaccharide is not affected in the same way as unbound protein.

Therefore, although freezing-drying offers many advantages, histochemical results must be interpreted with caution.

Experimental Work

Robertson (1941) postulated that the skin glands of *Helix* were organs for the excretion of excess calcium. The presence of a yellow pigment in the slime suggested that it, too, might be a waste product. An experiment was therefore carried out to see what properties of the secretions depend on the food of the animal.

Eighty-four snails which had been well fed with cabbage, rolled oats, and chalk were distributed between 7 glass tanks. Each series was kept under different conditions, thus:

Group A received a full diet of cabbage, rolled oats, and chalk.
Group B received carrot, rolled oats, and chalk.
Group C received cabbage and rolled oats.
Group D received rolled oats and chalk.
Group E received cabbage, chalk, and fibrin.
Groups F and G were starved.

Groups A to F were maintained at 18° to 21° C. They were moistened regularly, and given fresh food every 2 or 3 days; group G was kept in a refrigerator at 4° C.

The central part of the mantle-collar of one snail from each group was fixed, after 1, 2, 3, 4, 6, 8, 11, 15, 20, 42, 75, and 93 days, in formalin / lead acetate and embedded. Because quantitative results were desired, all the material was sectioned together and as far as possible stained together or for identical lengths of time.

The pigment could be observed in unstained sections; tests were needed for mucus, protein, and calcium. Those used were toluidine blue, Heidenhain's haematoxylin, and von Kossa respectively. It was obviously impossible to obtain precisely quantitative results in this way, but all the slides were examined in random order and the amounts of each kind of secretion estimated as normal, more, much more, less, and much less.

There were no apparent changes in the amounts of mucus present in these snails except a possible increase in those starved in the refrigerator. The only variation in protein was a slight drop in the starved, warm specimens and in those fed but without fresh plant food. The presence or absence of calcium in the food appeared to have no effect on the glands, although the snails readily ate the chalk; but starvation, especially in the refrigerator, produced a decrease in quantity. Perhaps the most interesting result was a decrease, particularly after a fortnight, of the amount of pigment in the glands from the group D snails—i.e. those which had had no fresh plant material in their diet. As well as this, however, there seemed to be a rather large amount in those kept in the refrigerator.

These results do not support Robertson's hypothesis that the glands excrete excess calcium; they coincide however with those of Wagge (1951), who regarded the shell-gland and digestive gland as stores. On this hypothesis the amount in the skin glands would be unrelated to diet.

The results from the snails kept in the refrigerator must be treated with caution since under such conditions metabolism would be slow and there would be a long period during which the materials already in the body at the beginning of the experiment could be utilized.

In summary, it seems that only the amount of pigment in the skin-glands bears any relationship to the diet of the animal.

This provokes the question of the nature of the pigment.

Yellow pigments in animal cells are likely to be either carotenoids, flavines, or flavones. Tests were carried out on fresh slime collected from irritated snails, since fixatives have a very marked effect on the colour, which is almost or quite missing from tissues treated with Susa, Zenker, or Carnoy, but is retained after formalin / lead acetate right through the hydration process, and after freezing-drying until the tissue reaches the alcohols.

The standard tests for carotenoids all gave negative results, and similarly those for flavines. However, the pigment shows the following characteristics of a flavone: it is water- and alcohol-soluble (hence its absence from most chemically-fixed material), is bleached by dilute acids, becomes brown with ferric chloride, deepens in colour when subjected to ammonia fumes, and evidently forms an insoluble yellow salt with lead as shown by the fixation in formalin / lead acetate.

The pigment has therefore been identified as a flavone. The histochemistry of the tissues showed at least most of the pigment to occur in glands whose contents were also positive with tests for protein. According to Fox (1953), carotenoids and flavines may be associated with protein, but he makes no mention of a combination with flavone. He states, however, that these substances are assumed to be taken into the animal body from plants. Flavones are very widely distributed in all organs of plants and this would be correlated with the observation that the quantity of pigment in *Helix* appears to vary with the amount of fresh plant food eaten. Fox recorded flavones only in insects and hydroids.

Reference was made in the introduction to the apparent change in the kind of slime released under different circumstances. To obtain slime, stimulation with electric shocks of constant duration and controlled frequency was used. The specimens were stimulated by holding the electrodes in contact with the body, as nearly as possible at the anterior and posterior extremities of the animal. Shocks were administered every minute, long enough for complete retraction into the shell, followed by re-emergence of the body. The voltages tested were 2, 5, 8, 10, 14, and 20, each snail receiving 4 similar shocks.

The type of slime released showed a direct correlation with the strength of the stimulus. At 2 to 5 volts it was usually colourless and markedly viscous—the normal slimy covering of a snail. At about 8 volts a whitish secretion sometimes, although not always, appeared—whitish because of the calcium carbonate granules present. Subsequently a yellow, much clearer slime was released, which was less viscous and rather watery in consistency; it was this type of secretion which accounted for the largest proportion of the total volume produced.

Dexheimer (1951) recorded two types of slime for *H. pomatia*: a frothy, clear secretion produced under normal conditions and on slight stimulation, and a sticky, creamy kind which appeared under long-continued or violent stimulation. As he did not specify the nature of the stimulus, it seems likely that he did not irritate sufficiently to release the third type of slime, if this species reacts in the same way as *aspersa*.

From the previous experiment it appeared that the slime released after irritation was more watery than the normal covering. In an attempt to prove this, snails were carefully cleaned of extraneous material clinging to them and wiped very gently with a clean, weighed glass slide. Each such slide was reweighed with its adherent slime and then left so that evaporation could occur until its weight was constant. This gave the water-content of the 'normal' slime as 86·1%.

After stimulation with 20 volts the thinner exudate was allowed to drip on to other slides and its water-content calculated as before. It was 96·3%. The results are statistically significant at well over the 1 in 1,000 level.

This proved therefore that the slime produced by an irritated snail is more watery than that normally released.

It was then desirable to determine, if possible, the relative amounts of the different kinds. A series of snails was stimulated 10 times with 20 volts while supported individually in funnels running into small specimen tubes of known weight; the thinner exudate dripped freely into the tubes, the thicker was removed from the creatures with forceps and added to the rest. Because of the movements of the snail in response to the shocks the slime was rather bubbly. It was weighed and left corked until most of the froth had disappeared and then the height in each tube was marked. After drying, the weight of the slime was redetermined. The dried residue was removed and water run into the tubes to the points marked from a graduated pipette, giving the volume of the slime.

If one knew the volume, total water-loss, and water-loss expected from each kind of slime, the relative amounts could be calculated. These figures gave a value of 98·6% for the proportion of thin yellow exudate in the total slime of the irritated animal. It must be emphasized, however, that these results are not very accurate, because of the difficulties in removing the thicker slime from the body.

Further properties were determined with slime collected from electrically stimulated snails.

The pH of this material, as shown by a series of indicator solutions, was between 8·5 and 9·0. Similar tests carried out on fresh-frozen sections gave the same figures, with some indication that the mucus was slightly more acid than the protein. This is in agreement with Prenant (1924), who recorded that the mucus (presumably meaning slime) of *H. pomatia* is slightly alkaline.

Although the protein-glands appear to discharge as part of a defence mechanism, their secretion is neither highly alkaline nor acidic. Tests with 35 people of different ages and both sexes suggest that there is no very strong flavour associated with the exudate.

Essex (1945) recorded a protein-splitting enzyme in the venom of some reptiles. On the chance that *Helix* might be similarly supplied, drops of slime were put on to sterile 10% gelatine films in Petri dishes. Controls with slime kept at 100° C for 10 min to activate any enzymes present were also set up.

After 24 h the gelatine below the untreated slime had been partly digested away, whereas it remained unaffected beneath the control drops.

The slime of *H. aspersa* has thus been shown to possess an active proteinase. Its importance is difficult to determine and it is unlikely to be strong enough to harm any predators. However, Russell (1954) reported that the epiphragm of this species appears to be dissolved away by mucus (presumably he meant the whole exudate), and the epiphragm is formed largely of protein. There would be little need for the breakdown process to be rapid and it may be that this is the function of the enzyme which has been detected.

The body of a snail normally appears clear of either bacterial or fungal growth, although constantly in contact with such organisms. Fischer (1948) suggested that the slime is actively antiseptic, and experiments were therefore devised to test this hypothesis.

Sterile Petri dishes of potato dextrose agar and peptone glucose agar were exposed overnight at ground level; half of the surface of each was then coated with the exudate from *H. aspersa* which had been collected with as little contamination as possible. Other plates with soil mixed with the agar as it cooled and therefore containing a wide variety of micro-organisms were treated similarly. After 3 days at 24° C in all cases fungi and bacteria were generally distributed over the plates and in 3 cases appeared more numerous on the side with the slime. The growth was so prolific, however, that similar tests with streaks of pure cultures of fungi and bacteria (species common in soil) counterstreaked with exudate were set up, but again the results showed no inhibition. Clearly, then, the slime does not act as an antiseptic.

To further the suggestion of the first experiment, that growth was actually enhanced, slime was smeared over half the surface of a series of plates of nutrient agar, which were then exposed at ground level overnight. Large numbers of colonies appeared and counts were made of those on and away from the smear. Analysed statistically at the 1 in 100 level, the population on the slime was bigger than that away from it.

There are two possible explanations—that the exudate retained spores settling on it more easily than the surface of the agar, and that it exerted a definite stimulatory effect. Although the first hypothesis is possible, since in the first trial the slime was administered after exposure to the air and the results appeared similar, this seems unlikely. The second may well be true. Perhaps the protein or carbohydrate offers extra nutrients to the micro-organisms. The possibility that the slime contains growth-promoting substances cannot be discounted.

DISCHARGE OF THE GLANDS

This is an aspect that nas received hardly any attention from previous workers.

Helix has an extensive system of haemocoel spaces and these might be assumed to take some part in causing discharge. Branches of these blood-spaces ramify throughout the body. The connective tissue of the mantle-collar is well supplied but the haemocoel rarely penetrates between the gland cells, although many cavities occur around their bases, and the same is true of the dorsal and lateral surfaces of the foot. In the sole of the foot, however, branches penetrate the whole glandular area (figs. 1–3).

Such a system could work by changing the pressure in the blood-spaces, which would in turn cause pressure on the glands and thus initiate their discharge. These changes in pressure would be brought about by movement of the animal, and on this would depend the discharge of the glands.

In the sole of the foot, changes in blood-pressure would result in the glands being compressed from all directions; the cell-bodies would respond to this pressure by ejecting their contents through their ducts to the surface. During locomotion a constant series of rhythmical contractions passes along the sole, producing rhythmical changes in pressure in the haemocoel, and on the glands, and a rhythmical discharge of their secretion. When the animal ceased to move, the secretion would cease to be released. From the functional point of view this would meet the snail's requirements, for only during locomotion would replenishment of the slime be necessary. In the sole of *H. aspersa*, therefore, the discharge of the glands could be accounted for quite adequately by changes in the blood-pressure.

Since the haemocoel does not penetrate so far between the glands in the mantle-collar and dorsal and lateral surfaces of the foot, pressure changes would constrict the glands less than in the sole. Although changes in body-form occur here too as the animal moves into and out of its shell, they are by no means so definite or continuous as on the sole. Thus, if dependent on blood-pressure, exudation would be spasmodic and perhaps not plentiful. However, since these surfaces are exposed to the air they need a good supply of exudate and experiment has shown that its volume and kind may vary with the situation in which the animal finds itself. It is therefore difficult to envisage blood-pressure alone as being responsible for discharge of the glands in these regions.

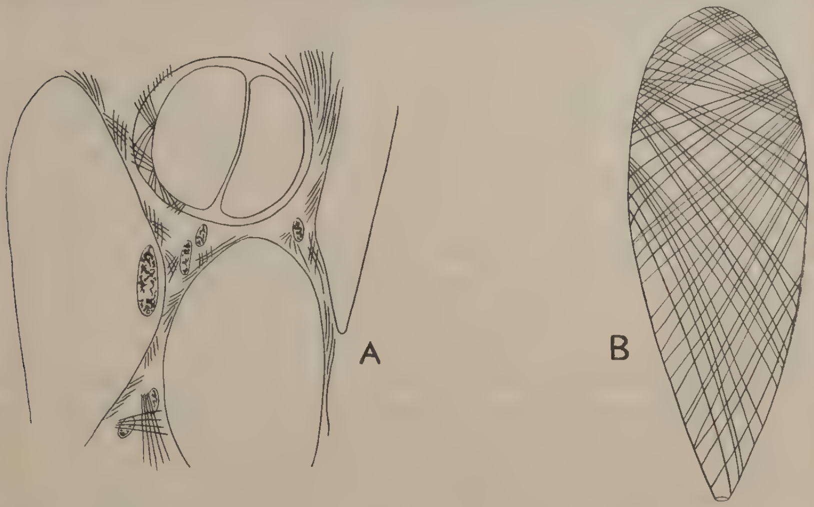

FIG. 4. A, part of a transverse section through the mantle of *H. aspersa*, showing muscle-fibres associated with the glands. B, a suggested reconstruction of the surface appearance of such a gland.

The majority of the glands found there are of a different type from those found on the sole: they are larger and have no distinct duct. The presence of muscle-fibres in the connective tissue around these cells has already been mentioned. When a surface view of part of the bounding membrane of one of these glands has been obtained it can be seen to be covered by an irregular network of fibres (fig. 4), which run obliquely around the inner part of the cell, and more longitudinally in the outer part. No sphincter or other structure has been seen on the duct.

Surface views of the network have been observed only round the protein- and the calcium-glands, although the appearance of sections is similar also round the large mucus-glands. The best material for investigating these fibres is that which has been frozen-dried. This process has a drastic effect on the mucus, causing it to be dispersed over the surrounding tissues and obliterating any vestiges of the original bounding membrane which might remain. Contraction of such a network round a cell of this shape would cause the gland to shrink not only in diameter but also in length, thus bringing about exudation. Although nothing is known about innervation of these structures, this method of discharge would seem to fit the situation better than blood-pressure, because the amount extruded could be controlled by nervous stimulation without any appreciable movement being necessary; and this could easily explain the increase in the volume of calcium and protein secreted when the animal is irritated.

No evidence of investing muscle-fibres has been seen in the glandular region of the sole: there the gland-cells are often found in clusters lying adjacent to one another, with apparently no space between in which fibres might be present.

Previous comment on discharge was made by Jones (1935), who, working on the mantle of *Anguispira alternans*, stated that 'Sometimes the large mucus glands may have a musculature enveloping the gland in a loose network of fibres, probably only a differentiation of closely adhering interglandular muscle fibres'. According to him similar structures are present in *H. pomatia* and *Polygyra thyroides*. Fretter and Graham (1949) recorded a basket-work of muscle-fibres around gland cells in the foot of pyramidellids, and they too thought they were involved in causing release of the secretion.

Thus one is led to the conclusion that the skin glands of *H. aspersa* are not all induced to discharge in the same way. It seems likely that in the sole blood-pressure is adequate to cause the release of the slime from the glands, but that in other regions, where most of the gland cells are club-shaped, this alone could not suffice, although, nevertheless, it may well play an important part. Here some glands, and probably all, are enveloped in a network of fine muscle-fibres, which on contraction cause exudation of the enclosed secretion.

Discussion

A certain amount of confusion exists in the terminology applied to mucoid substances, which makes precise interpretation of staining results difficult. This arises because some mucopolysaccharides may be firmly or loosely bound to varying amounts of protein, and although, according to Meyer (1945), chondroitin-sulphuric-acid and mucoitin-sulphuric-acid should properly be termed mucopolysaccharides even though they are firmly bound to protein, Hempelmann (1940) and Grishman (1952) described them as mucoproteins.

The amount of this associated protein and the way in which it is linked to the polysaccharide might be expected to affect the staining reactions of the carbohydrate; it may or may not itself be demonstrable. Thus it may be possible to be certain of the presence of protein by its staining reaction but it is not possible to be certain of its absence.

The standard test for a 'mucus' is the development of gamma metachromasia with a dye such as toluidine blue, but this result may be given by a variety of substances: thus Hempelmann (1940) regarded it as characteristic of most mucoproteins, including MSA and CSA, while Grishman (1948) listed only MSA and CSA. Lison and Mutsaars (1950) reported that nucleic acid may sometimes react metachromatically, but Sibatini (1952) stated that nucleic acids produce beta but not gamma metachromasia, and that the latter is characteristic only of mucopolysaccharides. Further detail was added by Hale in 1953, who regarded gamma metachromasia as indicative of polysaccharides with uronic acid as well as sulphate groups, or with polymeric phosphate groups.

Further comments on metachromasia include those of Landsmeer (1951–2), who discussed the importance of electrostatics, especially with respect to salt concentration, mono- and divalent ions suppressing staining; Sibatini (1952) agreed that electrostatic charges are significant. Grossfield (1954) noted that metachromasia changes on the death of the cell. According to Walton and Ricketts (1954) it is the attachment and dissociation of acidic radicles which is important and not polymerization of either the substrate molecules or the dye on the substrate, as was thought by Michaelis (1947) according to Pearse (1954). Clearly, then, it is impossible to be certain of the chemical significance of gamma metachromasia. It seems likely that both MSA and CSA always react in this way, although other substances may do likewise.

With regard to other methods, it should be noted that Steedman (1950) reported that all kinds of 'mucus' pick up alcian blue, and although Lison (1954) treats the test with caution because the mechanism is not understood, he too agreed that only acid mucopolysaccharides are stained and that mucoproteins and neutral mucopolysaccharides react faintly if at all. During this investigation, however, alcian blue has been taken up by a wider range of substances than has given gamma metachromasia.

Comments on the significance of positive PAS reaction also vary: McManus (1948) states that mucin and sometimes glycogen stain, Grishman (1952) regards it as demonstrating glycogen, some mucoproteins such as MSA but not others including CSA (using his terminology), and various other substances; Glegg, Clermont, and Leblond (1952) agreed that CSA is negative but some other mucins positive as well as a very few proteins. Against this must be put Hale (1953), who obtained a strongly positive result with free CSA.

It is difficult to distinguish between CSA and MSA, and attempts on *Helix* have been unsuccessful. CSA is usually a connective-tissue component, while MSA occurs in epithelia; in view of the apparent development of these glands from the connective tissue it would be interesting to know which occurs here.

It would seem likely that the secretion of the type A mucus-glands is an acid mucopolysaccharide containing no or only a small amount of protein. It may well be MSA or CSA. That from the B type of mucus-glands must be similar, since its reaction with toluidine blue and alcian blue is the same, but it picks up PAS far more readily, indicating some difference in molecular structure.

The sole-glands offer a problem. Their gamma metachromasia would seem to indicate an acid mucopolysaccharide character, although the result with Heidenhain's haematoxylin might mean that acidic protein is present, which is not found in the other kinds of mucus-gland. On Walton and Rickett's theory, protein should decrease the degree of metachromasia; yet this did not occur here. The safest description for this secretion is that it is a mucopolysaccharide probably associated with a considerable amount of protein, but not a mucoprotein as shown by the toluidine blue.

Similarly, the type D glands cannot be readily identified by their secretion. It is positive with PAS and alcian blue, but is not metachromatic with toluidine blue. This suggests, since it gives no reaction for protein, that it is a neutral mucopolysaccharide, although, according to Pearse (1954), alcian blue should give only a faint result in this case. The only other possibility is that the granules are composed of glycogen.

The functions of the various secretions must now be discussed. Of these lubrication is perhaps the most obvious.

Throughout the animal kingdom the main lubricating agents are mucopolysaccharides. They are found in the lining of the alimentary canal and genital tracts from worms to mammals, and in the skin from Cnidaria to Amphibia. Histochemically these secretions are similar to that found in the large type A mucus-glands and it is logical to assume, therefore, that the main action of this exudate is lubrication. The distribution of these glands in *Helix* coincides with the areas that would need an agent to reduce friction. They are most numerous on the median surface of the mantle skirt, an area where this would be of great importance.

Lubrication of a particularly well-controlled kind is essential on the sole of a snail because of the method of locomotion. Cilia cannot beat without fluid over them and one might expect that the muscular contractions which move the creature would result in a far less smooth kind of progression without the aid of some kind of mucopolysaccharide on the surface. Some of the mucus used in locomotion is released from the pedal-gland, which has not been considered here; it is logical to assume, however, that the great array of sole-glands also contributes to the supply. Ideally, the exudate should be fairly thin so that it would flow readily and offer no resistance to the waves of muscular activity. The protein associated with these glands may perhaps lower the surface tension of the mucus (which in other respects stains similarly to that of the type A glands) and enable it to flow more readily.

If the slime-trail from a snail which has moved over a slide is stained, the secretion from the sole-glands can be seen as a dense mass of extremely fine threads lying more or less parallel to one another. These are probably formed from the secretion droplets as the animal drags itself over the substratum. The very regular appearance of the trail suggests that the glands release their exudate gradually and more or less continuously. This would be expected if, as has been suggested, discharge is caused by changes in the blood-pressure as the animal moves. In contradistinction, threads are not evident in slime smears from the other surfaces of the body, which appear far more patchy, as though the secretion was released more spasmodically. It may be mentioned here that patches of irregular shape and size which stain pale blue with toluidine blue and often have an oval, darker area within, are usually apparent as well as the exudate; these are probably epidermal cells or parts of spent gland cells. The latter is the less likely, since one fails to find the developmental stages of glands to replace them.

Arey and Crozier (1921) stated that adhesion in pulmonates is due to slime and one must therefore assume that the secretion of the sole-gland must also be used for this. Gray (1926) showed that calcium makes intercellular matrices more viscous. Calcium is present in small amounts in the sole and this might be adequate to change the viscosity of the sole-gland secretions. Far more is present in the sides of the foot, which would mean that a rim of thicker mucus might be found round the edges of the sole, which may help in adhesion.

Robertson (1941) considered the calcium in the glands of *Helix* to be an excretory product, but this has been shown to be erroneous. It appears that the granules are released more copiously when the animal is irritated, and this would suggest that it has some protective action. The very fact of mixing granules with a fluid would make that fluid thicker, and Gray showed that divalent cations increase the viscosity of some protein or mucopolysaccharide substances. If the same effect occurred here, it would produce a deeper layer of slime over the body. The absence of calcium-glands from the median surface of the mantle, where thick slime is not desirable and where predators are unlikely to attack, supports this hypothesis.

A clue to the function of the protein may be its release in response to stimulation, from which one might conclude that it has some defensive action. Tests of pH, taste, and the effect on micro-organisms do not support the idea of active defence or attack. However, it is a very watery secretion, of much lower surface tension than the mucus, and this is probably the keynote of its action. A snail that has been irritated by powdered metaldehyde, the well-known slug poison, responds by extruding vast quantities of this protein secretion; the result is that the slime, carrying the power, runs off the body, and it seems likely that this is the normal mode of action.

It is difficult to decide the function of the two other types of mucus-gland, B and D; neither occurs abundantly, and their action may well be supplementary to that of the others producing mucopolysaccharides.

Hogben and Kirk (1944–5) suggested that the slime is important in temperature regulation by evaporation. Certainly it is far from hygroscopic and its production must cause considerable dehydration of the animal, but *Helix* is rarely found in dry or hot situations where evaporation would have its maximum effect on temperature regulation.

One final function of the slime must be mentioned—that of the formation of the epiphragm, which is thought to be produced from the combined secretions from the ventral surface of the mantle-collar. The presence of a proteinase in the slime and its possible link with the dissolution of the epiphragm has already been discussed.

I am very grateful to Professor A. Graham for providing me with facilities for research, including the use of the Edwards's freeze-drier, in his Department of the University of Reading, and especially for numerous discussions during the progress of the investigation. I am also deeply indebted to the Mrs. Smith Trust Fund for a grant which enabled me to continue the work.

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Histological Changes in the Gut of *Mitopus morio* (Phalangiida) during Protein Digestion

By JOHN PHILLIPSON

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SUMMARY

1. The description of the gross morphology of the gut of *Mitopus morio* agrees with Kästner's (1933) description for *Opilio parietinus* De Geer except that 4 pairs of lateral openings lead from the mid-gut into the diverticula and not 3 as previously recorded.
2. The two main types of diverticular cell in *M. morio* are digestive and secretory. There is thus agreement with the structures present in spiders, scorpions, and other harvest-spiders.
3. The mid- and hind-gut epithelia both possess a single cell-type. That of the mid-gut is absorptive and digestive, whereas that of the hind-gut secretes a membrane round the faeces to form a pellet.
4. During protein metabolism some extracellular digestion occurs in the lumina of the diverticula and mid-gut. The necessary enzyme or enzymes are produced by the diverticular secretory cells and are replenished within 24 h.
5. Extracellular digestion is followed by partial or complete intracellular digestion in the diverticular digestive cells, which then cut off either digestive cell apices, containing partially digested protein globules, or digestive cell faeces which contain excretory material.
6. Further digestion of the protein globules of the digestive cell apices occurs in the mid-gut cells, which then produce mid-gut cell faeces that are voided with the digestive cell faeces and indigestible food remnants.

INTRODUCTION

THERE is a close similarity in the gut structure of all Arachnida (Grassé, 1949) and a similar agreement in function of the cell components of the gut might well be expected. To some extent this has been established. Thus Kästner (1935) and Frank (1937) showed the two main types of cell in the mid-gut diverticula of harvest-spiders to be digestive and secretory, which agrees with the descriptions by Millot (1926) for spiders and Pavlovsky and Zarin (1926) for scorpions. However, Gilbert (1952) working on Chelonethi (pseudo-scorpions) showed that the diverticular cells were of two types, digestive or excretory. The problem therefore arises whether there has been a misinterpretation of cell function in the other groups. In view of these divergences, the histology of the gut system in harvest-spiders has been re-investigated by Gilbert's (1952) techniques. This has entailed a description of the general morphology of the gut in *Mitopus morio* and a study of the histological changes occurring during protein digestion.

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MATERIALS AND METHODS

The adult *M. morio* (F) used in this work were collected by hand in the vicinity of Durham City and maintained in the laboratory in accordance with Phillipson's (1960) directions.

The general morphology of the gut was determined by dissecting specimens (fixed in 95% alcohol) under a stereoscopic microscope with fine needles. Details were obtained from serial sections of animals fixed in Susa, embedded in paraffin wax (melting-point 54° C), cut at 8 μ, and stained in haematoxylin and eosin.

Changes in the gut epithelia during protein digestion were determined on animals kept individually, each fed on one *Scopeuma stercoraria* female and then starved for 3 days. They were then given a further feed of the same food and fixed at intervals of ½ h, 2 h, 6 h, 12 h, 18 h, 1 day, 2 days, 4 days, and 6 days after feeding. At least two individuals were used for each observation and Gilbert's (1952) techniques were employed.

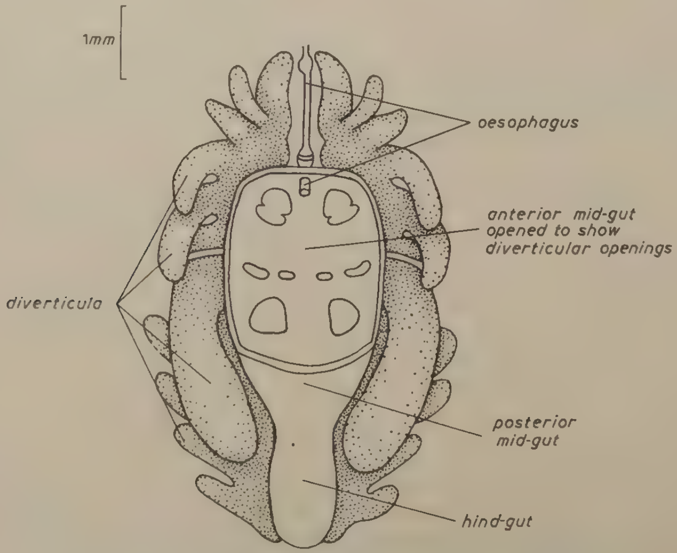

FIG. 1. *Mitopus morio.* General morphology of the gut (ventral view).

MORPHOLOGY OF THE GUT

Kästner (1934) showed that the morphology of the gut of harvest-spiders differed between families. The Phalangiidae, to which *M. morio* belongs, has the most complex system of mid-gut diverticula. Fig. 1 shows that the narrow, chitin-lined oesophagus expands into a capacious mid-gut which is divided into anterior and posterior regions. It is clear from both sections and dissections of *M. morio* that 4 pairs of openings lead into the diverticula from the anterior mid-gut and not 3 pairs as stated by Kästner (1933) for *Opilio parietinus* De Geer and *Phalangium opilio* L. The posterior region is not perforated by diverticular openings, and the hind-gut, divided from the mid-gut by a constriction, is short.

Histology of the Gut

The anterior mid-gut is lined with a uniform columnar epithelium composed of one cell-type. The posterior mid-gut, although lined with cells of the same size and structure as those of the anterior mid-gut, has its epithelium produced into 'papillae' which project into the lumen. In the diverticula (fig. 2, A) two main types of cell can be distinguished, both differing from those in the mid-gut epithelium. One type, the digestive cell, is binucleate and occurs in the ratio of approximately two to every secretory cell. A uniform columnar epithelium of yet another cell type lines the hind-gut.

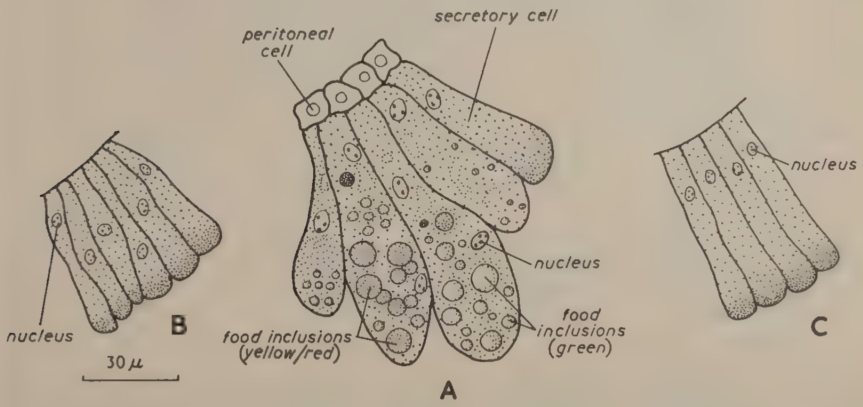

FIG. 2. A, wall of diverticular mid-gut. B, mid-gut cells. C, hind-gut cells. All 30 min after feeding. Susa: haemalum / acid fuchsin / metanil yellow / light green.

Table 1 summarizes the results obtained during protein digestion by staining with the modified quadruple method of Gilbert (1952). The reagents are haemalum which stains nuclei blue, light green which stains the ground cytoplasm green, and metanil yellow and acid fuchsin, both of which stain cell inclusions. The inclusions, which are believed to be protein, show a progressive change in their staining reaction from green to yellow-red, to red, and finally red-brown. The changing colour reaction is generally accepted to indicate different stages in protein metabolism.

At ½ h and 2 h after feeding, although columnar diverticular digestive cells occurred (fig. 3, A), they were of the type described as 'young cells' by Frank (1937) and not equivalent to the later 'columnar' cells.

Sections stained with Heidenhain's iron haematoxylin, apart from coloration, gave identical results with those described above, except that in the Heidenhain series rod-shaped structures about 12 μ long and small globules some 0·75 μ in diameter occurred, at all feeding stages, in the cells surrounding that part of the oesophagus which projected into the mid-gut lumen. The

TABLE I. *Summary of results obtained from sections stained by the quadruple method*

Cell type	*Size and shape of cell*	*Cell appearance at intervals (in hours) after feeding*								
		½	2	6	12	18	24	48	96	144
Diverticular secretory	60 μ high 25 μ wide Ovoid when fully developed	Fig. 2, A No inclusions	Fig. 3, A No inclusions	Fig. 4, A Small yellow/red globules	Numerous small yellow/red globules	Fig. 6, A Numerous large yellow/red globules	Filled with yellow/red globules	Fig. 7, A Filled with yellow/red globules	Packed with yellow/red globules	Fig. 8, A Packed with yellow/red globules
Diverticular digestive (d.d.)	90–100 μ high 30–35 μ wide Clavate	Fig. 2, A Mainly green and yellow/red globules	Fig. 3, A Mainly yellow/red and red globules	Fig. 4, A Yellow/red, red or red/brown globules in apical vacuole	Yellow/red, red or red/brown globules in apical vacuole	Fig. 6, A Red or red/brown globules in apical vacuole	Red or red/brown globules and granules in apical vacuole	Fig. 7, A Red/brown globules and granules in apical vacuole	Red/brown granules in apical vacuole	Fig. 8, A Red/brown granules in apical vacuole
	60–75 μ high 12–15 μ wide Columnar	See text	See text	Fig. 4, A No inclusions d.d. cell apices present	No inclusions d.d. cell apices present	Fig. 6, A No inclusions d.d. cell apices present	No inclusions Few d.d. cell apices and few d.d. cell faeces present	Fig. 7, A No inclusions Few d.d. cell apices but many d.d. cell faeces present	No inclusions Many d.d. cell faeces present	Fig. 8, A No inclusions Many d.d. cell faeces present
Mid-gut (m.g.)	45–60 μ high 6–12 μ wide Columnar	Fig. 2, B No inclusions	No inclusions	Fig. 4, B A few red globules Apical vacuoles	Fig. 5 Numerous red globules	Fig. 6, B Red globules aggregated in apical vacuoles	Red and red/brown globules in vacuoles	Fig. 7, B Red/brown particles in apical vacuoles Cell faeces present	No inclusions m.g. cell faeces present	Fig. 8, B No inclusions Cell faeces present
Hind-gut (h.g.)	60–75 μ high 15 μ wide Columnar	Fig. 2, C h.g. cell apices absent	Fig. 3, B h.g. cell apices present	h.g. cell apices present	h.g. cell apices present	h.g. cell apices present	h.g. cell apices present	h.g. cell apices present	h.g. cell apices present	h.g. cell apices present

Explanation of the terms used in table 1:

Diverticular digestive cells. d.d. cell apices = portion separated from the digestive cell and containing variously coloured globules (fig. 4, A); d.d. cell faeces = spheres separated from the digestive cells and containing red/brown stained granules (fig. 7, A).

Mid-gut cells. m.g. cell faeces = small spheres separated from the mid-gut cells and containing red/brown stained particles (fig. 7, B).

Hind-gut cells. h.g. cell apices = small spheres separated from the hind-gut cells and stained pink/brown (fig. 3, B).

rods and globules were also present in many of the mid-gut cells of animals which had been starved for 6 days (fig. 9, p. 226).

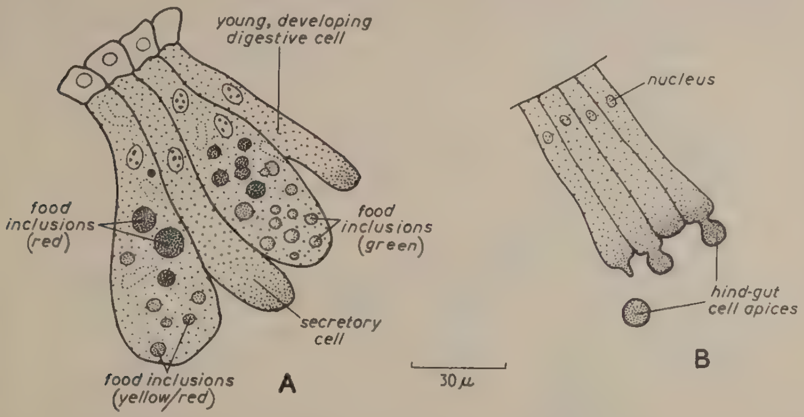

Fig. 3. A, one secretory and 3 diverticular digestive cells. B, hind-gut cells. Both 2 h after feeding. Susa: haemalum / acid fuchsin / metanil yellow / light green.

Gut Contents

Unlike spiders and pseudoscorpions, which allow only liquid food to enter the gut, the harvest-spiders take in small solid particles as well as liquid.

Thirty minutes after feeding. The lumina of both mid-gut and diverticula were filled with a mixture of solid and liquid. The solid material was not wholly digestible because fragments of arthropod exoskeleton were present, although the peripheral region of the food-mass was wholly liquid. The hind-gut lumen contained fragmented food and a few digestive cell faeces which lay centrally; it was assumed that the latter were from a feed previous to the experimental one.

Two hours after feeding. The lumina of the diverticula contained only liquid food ('*Brei*'), whereas the mid-gut contents consisted of a central mass of solid with a wide peripheral band of *Brei.* The hind-gut lumen contained solid particles, most of which were indigestible exoskeleton, encircled by a few hind-gut cell apices.

Six hours after feeding. The diverticular lumina contained not only *Brei* but also digestive cell apices (fig. 4, A), and the mid-gut lumen had solid particles and a wide peripheral band of *Brei* plus a few digestive cell apices. In the hind-gut lumen there was some *Brei* in addition to the solid particles and hind-gut cell apices.

Twelve hours after feeding. The diverticular lumina were filled with *Brei*, which had a finely granulated rather than wholly liquid appearance. Numerous digestive cell apices and a few digestive cell faeces were present. The mid-gut lumen contained a central mass of solid particles surrounded by granular *Brei* and numerous digestive cell apices. In the hind-gut the solid particles plus granular *Brei* were surrounded by hind-gut cell apices which formed a membrane around the hind-gut contents.

Eighteen hours after feeding. Only a few digestive cell apices were interspersed with the granular *Brei* in the diverticular lumina, but numerous digestive cell faeces were present. In the mid-gut lumen solid particles were no longer obvious, and the granular *Brei* plus the few digestive cell apices (fig. 6, A) and faeces which were present had formed into a pellet-like mass. The hind-gut lumen contained in addition to solid particles a few centrally placed digestive cell apices and faeces, the whole surrounded by a membrane formed by the hind-gut cell apices.

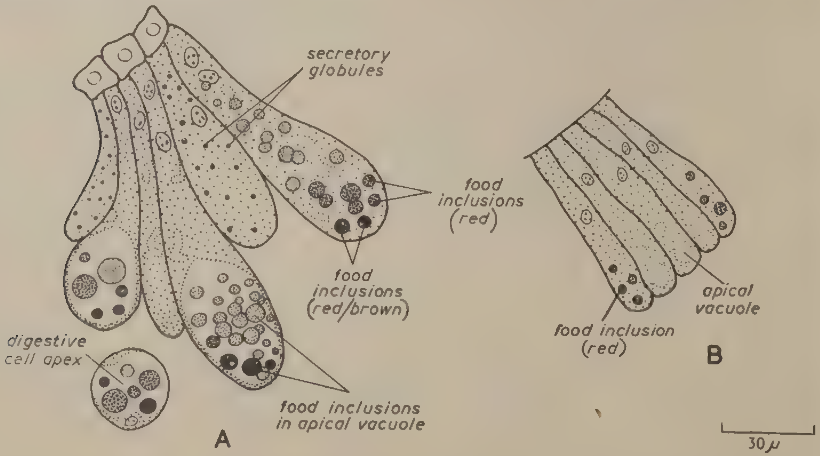

Fig. 4. A, two secretory and 4 diverticular digestive cells; one of the latter has shed its apex. B, mid-gut cells. Both 6 h after feeding. Susa: haemalum / acid fuchsin / metanil yellow / light green.

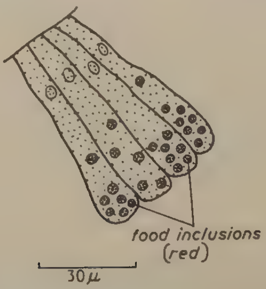

Fig. 5. Mid-gut cells 12 h after feeding. Susa: haemalum / acid fuchsin / metanil yellow / light green.

One day after feeding. Very few digestive cell apices and a few digestive cell faeces mixed with *Brei* were present in the diverticular lumina. The mid-gut lumen contained digestive cell apices and faeces, the former in a central and the latter in a peripheral position relative to the granular *Brei*. In the hind-gut lumen were digestive cell apices and faeces as well as granular *Brei*.

Two days after feeding. The diverticular lumina still contained granular *Brei* but no digestive cell apices and only a few digestive cell faeces (fig. 7). The mid-gut cell lumen did contain digestive cell faeces, centrally placed in the granular *Brei*, and mid-gut cell faeces in a peripheral position. The faecal pellet in the hind-gut was similar to that described for 24 h after feeding, but less indigestible food material was present.

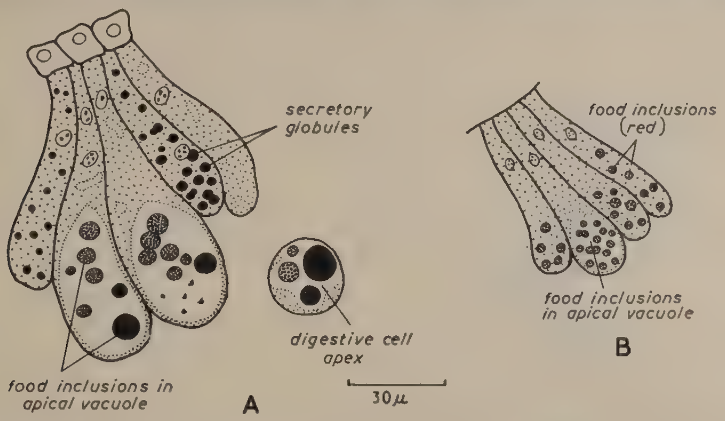
secretory globules
food inclusions (red)
digestive cell apex
food inclusions in apical vacuole
B
food inclusions in apical vacuole
A
30μ

FIG. 6. A, two secretory and 3 diverticular digestive cells; one of the latter has shed its apex. B, mid-gut cells. Both 18 h after feeding. Susa: haemalum / acid fuchsin / metanil yellow / light green.

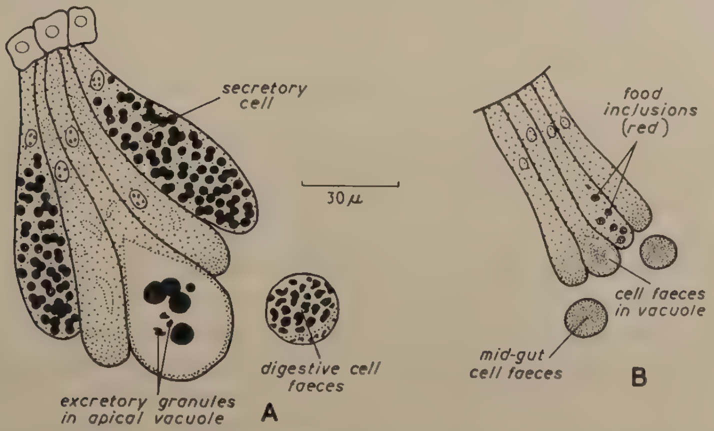

FIG. 7. A, two secretory and 3 diverticular digestive cells. Two of the latter have shed their cell faeces. B, mid-gut cells, two of which have shed their cell faeces. Both 48 h after feeding. Susa: haemalum / acid fuchsin / metanil yellow / light green.

Four days after feeding. Little or no granular *Brei* was present in the diverticular lumina and only a few digestive cell faeces. The contents of the mid-gut lumen were as described for 2 days after feeding. The hind-gut contained mid-gut cell faeces in addition to digestive cell faeces, but no indigestible solid particles were present.

Six days after feeding. The contents of the diverticular lumina were as described for 4 days and the mid-gut lumen no longer contained granular *Brei*, only numerous mid-gut cell faeces. The hind-gut lumen was also comparatively empty and contained a little granular *Brei*, a few digestive cell faeces, and many mid-gut cell faeces.

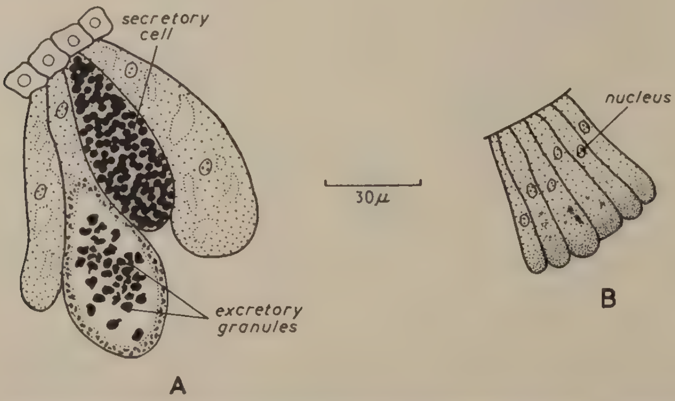

FIG. 8, A, one secretory and 3 diverticular digestive cells, one of which still contains excretory granules. B, mid-gut cells. Both 6 days after feeding. Susa: haemalum / acid fuchsin / metanil yellow / light green.

DISCUSSION

The examination of the gross morphology of the gut of *M. morio* agrees in general with the description given by Kästner (1933) for the closely related *O. parietinus* and *P. opilio*, but it is evident that 4 and not 3 pairs of lateral diverticular openings occur in the anterior mid-gut.

In agreement with Frank (1937), both particulate and liquid food was taken into the lumina of the diverticula and mid-gut of *M. morio*, and within 30 min after feeding the diverticular secretory cell contents, which are visible in starved animals, had disappeared, presumably emptied into the diverticula and mid-gut lumina. This establishes that one of the cell types in the diverticula is secretory as described by Millot (1926) for spiders, Pavlovsky and Zarin (1926) for scorpions, and Frank (1937) for harvest-spiders, and not excretory as found in pseudoscorpions by Gilbert (1952). Gilbert's main reason for assigning an excretory rather than secretory role to the second type of diverticular cell in pseudoscorpions (even though the products of the cells were in some respects similar to those of the secretory cells of spiders) was that there seemed to be no direct relationship between the activity of the digestive cells and that of the 'excretory' cells. Further, Gilbert (1952) states that Malpighian tubules are absent in pseudoscorpions, whereas they are present in spiders, scorpions, and harvest-spiders. This suggests that the lack in pseudoscorpions may be associated with the presence of excretory rather than secretory cells in the diverticula. However, it is possible that Gilbert (1952) was mistaken in his conclusions about the function of the diverticular cells in the Chelonethi in that harvest-spiders, like pseudoscorpions, do not possess Malpighian tubules (Berland, 1949) and yet *M. morio* still has diverticular secretory cells.

Unlike spiders (Millot, 1926), the globules of the secretory cells in *M. morio* were not visible as morular masses in the gut lumina. At the same time the periphery of the food-mass within the gut was in a liquid state (*Brei*), indicating that extracellular digestion was taking place, a feature that both Kästner (1935) and Frank (1937) noted. The *Brei* was taken up by the diverticular digestive cells and formed protein globules of varied size. To judge by the changing staining reaction of the globules over a period of 24 h, they were undergoing further digestion. The colour changes observed in the diverticular digestive cell inclusions agreed closely with those reported in the Chelonethi by Gilbert (1952).

Six hours after feeding, digestive cell apices were cut off into the diverticular lumina. This compares with Frank's (1937) findings that cell apices cut off as early as 8 h after feeding differed from the later 'cell faeces' in that they contained not only excreta but also globules. Gilbert (1952) found digestive cell apices cut off in the Chelonethi quite soon after feeding but the contents had broken down into 'cell faeces' without the cellular inclusions having gone through the normal acid-fuchsinophil stage. At this time, in *M. morio*, small globules first appeared in the diverticular secretory cells. It was also at this stage, when digestive cell apices entered the mid-gut lumen, that small cellular inclusions plus apical vacuoles appeared in the mid-gut cells. The presence of vacuoles in the mid-gut cells (not noted by earlier workers) 6 h after feeding cannot be explained but may have some connexion with the change in appearance of the *Brei* from a liquid to a granular stage, as observed 12 h after feeding when the vacuoles had disappeared.

The number of mid-gut cell inclusions increased as the number of digestive cell apices in the mid-gut decreased, and it is assumed that the mid-gut cells took up the partially digested material of the digestive cell apices. Kästner (1935) believed that the digestive cell apices were probably stored in the gut. However, according to Frank (1937), the digestive cell apices on reaching the mid-gut lumen lose their contained globules, and he suggested that they were absorbed by the mid-gut cells. He noted also that small globules of unknown origin and development, but presumably metabolic products, were present in the gut cells in considerable numbers during active digestion. The relationship between an increased number of mid-gut cell inclusions and the disappearance of digestive cell apices was not realized by Frank (1937), and it was stated that no mid-gut cell faeces were produced. It is evident from the present work that mid-gut cell faeces were produced about 24 h after feeding and that the mid-gut cells complete the digestion of the partially denatured protein of the digestive cell apices. This procedure differs from that in the Chelonethi, where, according to Gilbert (1952), the digestive cell apices/faeces remained in the gut lumen as faeces, but the 'excretory cell' apices were taken up in the syncytial epithelium of the post-diverticular mid-gut and there converted into amorphous material accompanied by the secretion of a similar material into the lumen.

While mid-gut cell digestion was taking place in *M. morio*, further activity occurred in the diverticular digestive cells, and digestive cell faeces, equivalent to the cell faeces of both Frank (1937) and Gilbert (1952), were produced. The digestive and mid-gut cell faeces became mixed with the indigestible material of the mid-gut contents and were passed into the hind-gut.

In the hind-gut a membrane was formed round the faecal material by the hind-gut cell apices as described by Frank (1937). The formation of the first faecal membrane at 12 to 18 h after feeding agrees with Phillipson's (1960) findings that the first faecal pellet from a given food is voided 15·5±1·5 h after feeding in males and 19·7±3·2 h in females.

The presence of rod-shaped structures and globules during starvation in most of the mid-gut cells in sections stained with Heidenhain's haematoxylin (fig. 9) is not understood. Frank (1937) noted the rods in the oesophageal cells only and suggested that their function might be either skeletal or food storage. Their presence in the mid-gut cells during starvation and at no other time does not support either theory but suggests a possible excretory function.

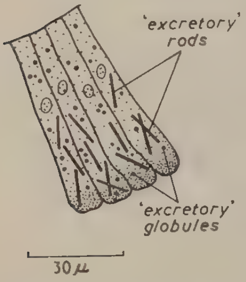

FIG. 9. Hind-gut cells 6 days after feeding. Susa: Heidenhain's iron haematoxylin.

I should like to thank Prof. J. B. Cragg for his help in the writing of this paper.

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The Turnover of Melanin in *Xenopus laevis* treated with Phenylthiourea

By R. T. SIMS

(*From the Department of Anatomy, University of Cambridge*)

With one plate (fig. 2)

SUMMARY

Four sets of experiments were performed in which developing *Xenopus laevis* were treated with 0·1% phenylthiourea. The animals used in experiments 1 and 2 were taken from a single batch of eggs, those in experiment 3 from another batch of eggs, and those in experiment 4 from a third batch. A suitable control group of untreated tadpoles was arranged for each experiment. In experiment 1 gastrulae were reared in a solution of phenylthiourea, which inhibited melanogenesis. In experiment 2 tadpoles, which possessed melanophores containing melanin, were kept in a solution of phenylthiourea for 6 weeks. No turnover of melanin was detected in these animals. Experiment 3 was carried out on tail regenerates. It showed that melanogenesis is inhibited by phenylthiourea and that no turnover of melanin occurs during treatment with this drug. In experiment 4 animals which had almost completed metamorphosis showed no evidence of turnover of melanin when treated with phenylthiourea.

The arrest of development of the tadpoles and the histological changes in the thyroid glands which occurred during treatment were used as evidence that phenylthiourea reached the tissues of the experimental animals.

INTRODUCTION

MELANOPHORES of vertebrates effect colour changes of the integument in two ways, by dispersion and concentration of the pigment granules within the melanophores and by alteration of the amount of pigment formed and released into the integument. The ability of melanophores in Anura to disperse and concentrate their pigment is well established, but I have been unable to find any description of the release of pigment into the anuran integument later than that of Ehrmann (1885). Experiments with inhibitors of melanogenesis indicate that there is a turnover of melanin during the development of anurans. Lewis (1932) found that tadpoles of *Rana sylvatica* reared in solutions of indophenol dyes lost the pigment of their melanophores. Richter and Clisby (1941) reported that black rats treated with phenylthiourea became grey after 27 to 58 days, and Lynn (1948) showed that embryos of *Eleutherodactylus ricordii* raised in solutions of phenylthiourea lost the melanin of the skin and pigmented coat of the eyes in 5 or 6 days. In the same paper reference was made to unpublished experiments in which tadpoles of *R. pipiens* reared in a solution of phenylthiourea lost melanin from the skin but not from the eyes. Millott and Lynn (1954) reported experiments with embryos of *E. martinicensis*, in which the melanin of the skin, iris, and retina disappeared after 5 days' treatment in solutions of phenylthiourea.

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The mode of action of phenylthiourea is uncertain but it is generally agreed that it inhibits melanogenesis by a direct effect on the melanophore. If it has no effect on the pigment itself, loss of melanin from melanophores after 5 days' treatment represents a rapid turnover for a substance which chemically is only affected by strong oxidizing agents. The experiments reported here were undertaken to investigate this paradox. It was hoped to estimate the rate of turnover of melanin and the fate of the granules which disappear in an anuran tadpole readily available in the laboratory, and to observe the effect of phenylthiourea on development of the tadpole and the histology of the thyroid gland.

MATERIAL AND METHODS

Chorionic gonadotrophin was used to induce amplexus and ovulation in adult *Xenopus laevis*. The animals were reared and all experiments carried out in glass vessels kept on a matt black surface in a cupboard, the temperature of which was maintained at 20° C by a thermostat. Environmental factors that affect development, such as temperature, light, degree of crowding, and amount of food given per animal, were all standardized. The tadpoles were fed with a suspension of nettle-powder in water and when necessary their water was aerated. The methods of rearing and staging the animals were those recommended by Nieuwkoop and Faber (1956).

Preliminary experiments showed that phenylthiourea at a concentration of 0·01% was required in the water to prevent the formation of melanin in the tadpoles and that fatalities occurred at intervals during treatment at this dose. A concentration of phenylthiourea low enough to avoid fatalities did not completely inhibit melanogenesis. The water and treatment-solutions were changed daily.

The course of the experiments was followed by anaesthetizing tadpoles with a 1:5000 solution of M.S. 222 and observing pigmentation both with the naked eye and with a dissecting microscope; they were then returned to their respective vessels to recover. For more detailed examination specimens were fixed in this solution:

formalin (commercial solution of formaldehyde, about 40%) 5 ml
acetic acid (glacial) 0·5 ml
distilled water up to 100 ml

The specimens were dehydrated in ascending grades of alcohol and passed through xylene into Canada balsam. When tails were mounted whole, they were stained in 1% aqueous neutral red, washed in distilled water, transferred on to a microscope slide, dehydrated in an oven at 39° C, and then cleared in xylene before being mounted in balsam. This method of dehydration greatly reduces the thickness of the tail and allows a search for melanin granules to be made with a 3·6-mm oil-immersion objective. Serial paraffin sections 10 μ thick were prepared and stained with Weigert's haematoxylin and eosin, but it was found that the best method for demonstrating melanin was to stain in bulk with borax carmine.

Four sets of experiments were conducted and will be outlined here; details will be presented with the observations. The animals were 'staged' at the beginning and end of the experiments; that is to say, the stage of development was determined and noted down as a number, in accordance with the scheme published by Niewkoop and Faber (1956). At the same time observations were made on the histology of their thyroid glands. The animals used in experiments 1 and 2 were taken from a single batch of eggs, those in experiment 3 from another batch of eggs, and those in experiment 4 from a third batch of eggs.

Experiment 1 was designed to demonstrate the inhibition of melanogenesis by 0·01% phenylthiourea. Eggs were reared in tap-water and in tap-water containing 0·01% phenylthiourea.

Experiment 2 was designed to demonstrate the turnover of melanin by treating one set of tadpoles with phenylthiourea to inhibit melanogenesis while another set was kept in tap-water as controls.

Experiment 3 was designed to demonstrate both the inhibition of melanogenesis and the rate of turnover of melanin of known age in tadpoles at the same stage (later than that of experiment 2).

Experiment 4 was designed to demonstrate the turnover of melanin in animals which had almost completed metamorphosis.

OBSERVATIONS

Experiment 1. Early in the morning 150 eggs were taken from a batch laid overnight and divided into two groups. 50 were reared in tap-water while the other 100 were reared in a solution of phenylthiourea in tap-water. At this time the eggs were undergoing gastrulation: formation of the neural plate and its derivatives had not begun.

During the following days a difference in colour between the control and treated animals became progressively more marked. The control group developed deeply pigmented melanophores around the nasal pits, on the dorsum of the trunk, over the tail musculature, and in the meninges, parietal peritoneum, and pronephros. The pigmented coat of the eye was a dense black. The treated animals were almost transparent, except the coiled intestine which was dark brown and opaque even before feeding had started. No melanophores containing black pigment could be found when the animals were examined alive and the pigmented layer of the eye had a faint yellow appearance.

In order to confirm that the distribution of melanophores was similar in the treated animals and in the controls, several of the treated animals were transferred to tap-water alone on the 9th day of the experiment. Melanin began to appear after 2 days. Its distribution was the same as that in the controls and it did not appear in any one region before another.

The experiment was terminated on the 20th day. All the animals were staged. The results are presented in a histogram (fig. 1), from which it can be seen that the number of survivors in each group was about the same, and the majority of the treated group were one stage behind the control group in development.

In the sectioned material the thyroid glands of the control group consisted of cell-nests showing the beginning of follicle formation, while in the treated group the cell-nests were smaller and no evidence of follicle formation was found.

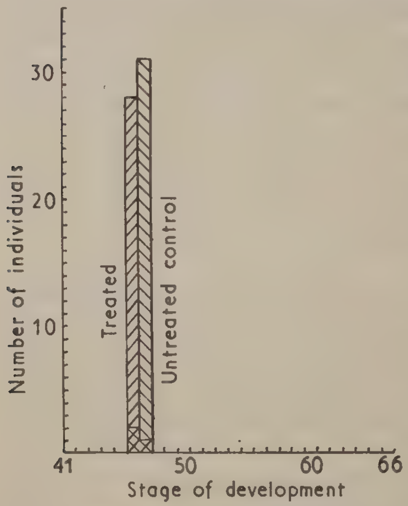

FIG. 1. *Experiment 1.* Histogram showing the number of *Xenopus* tadpoles at each stage of development, 20 days after laying. ▨ treated with phenylthiourea; ▧ untreated control group.

The melanophores of the control group contained dark black melanin. In the whole mounts melanophores were seen in the region of the nasal pits, on the medial wall of the otic capsule, on the superficial surface of the axial muscles of the trunk and tail, on the pronephros, over the parietal peritoneum, and scattered over the head immediately beneath the epithelium and in the dorsal and ventral fins. The melanin of the eye rendered it pitch-black (fig. 2, A). These observations were readily confirmed on the sectioned specimens, in which an abundance of melanophores was found also on the lungs, in the mesentery of the intestine, and among the muscle-fibres of the axial muscles. Cells packed full of melanin granules were scattered throughout the

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FIG. 2 (plate). A, *Experiment 1.* Oblique section through the pigmented coat of the eye of a tadpole of *X. laevis* at stage 47, reared in tap-water for 20 days after the eggs were laid. An abundance of melanin is present.

B, *Experiment 1.* Oblique section through the pigmented coat of the eye of a tadpole of *X. laevis* at stage 46, reared in a solution of phenylthiourea for 20 days after the eggs were laid. Melanin is lacking.

C, *Experiment 2.* Oblique section through the pigmented coat of the eye of a tadpole of *X. laevis* at stage 47, after it had been kept in a solution of phenylthiourea for 6 weeks from the time it reached stage 46 or 47 in development. No reduction in the amount of melanin is apparent. Compare A.

D, *Experiment 3.* Area of tail-regenerate of group A kept in tap-water for 8 weeks after amputation of the tail. There is an abundance of melanin in the melanophores.

E, *Experiment 3.* Area of tail-regenerate of group C which was allowed to develop a little melanin before being treated with phenylthiourea for 6 weeks. No loss of melanin was detected.

F, *Experiment 3.* Area of tail-regenerate of Group B which was treated with phenylthiourea continuously for 8 weeks after amputation of the tail. Melanophores cannot be seen owing to the absence of melanin.

G, *Experiment 4.* Part of a section of the hind limb of an untreated control animal, showing melanophores among the cells of the epidermis and beneath it.

H, *Experiment 4.* Part of a section of the hind limb of an animal treated with phenylthiourea for 6 weeks, showing melanophores among the cells of the epidermis and beneath it containing the same amount of melanin as those in the control group.

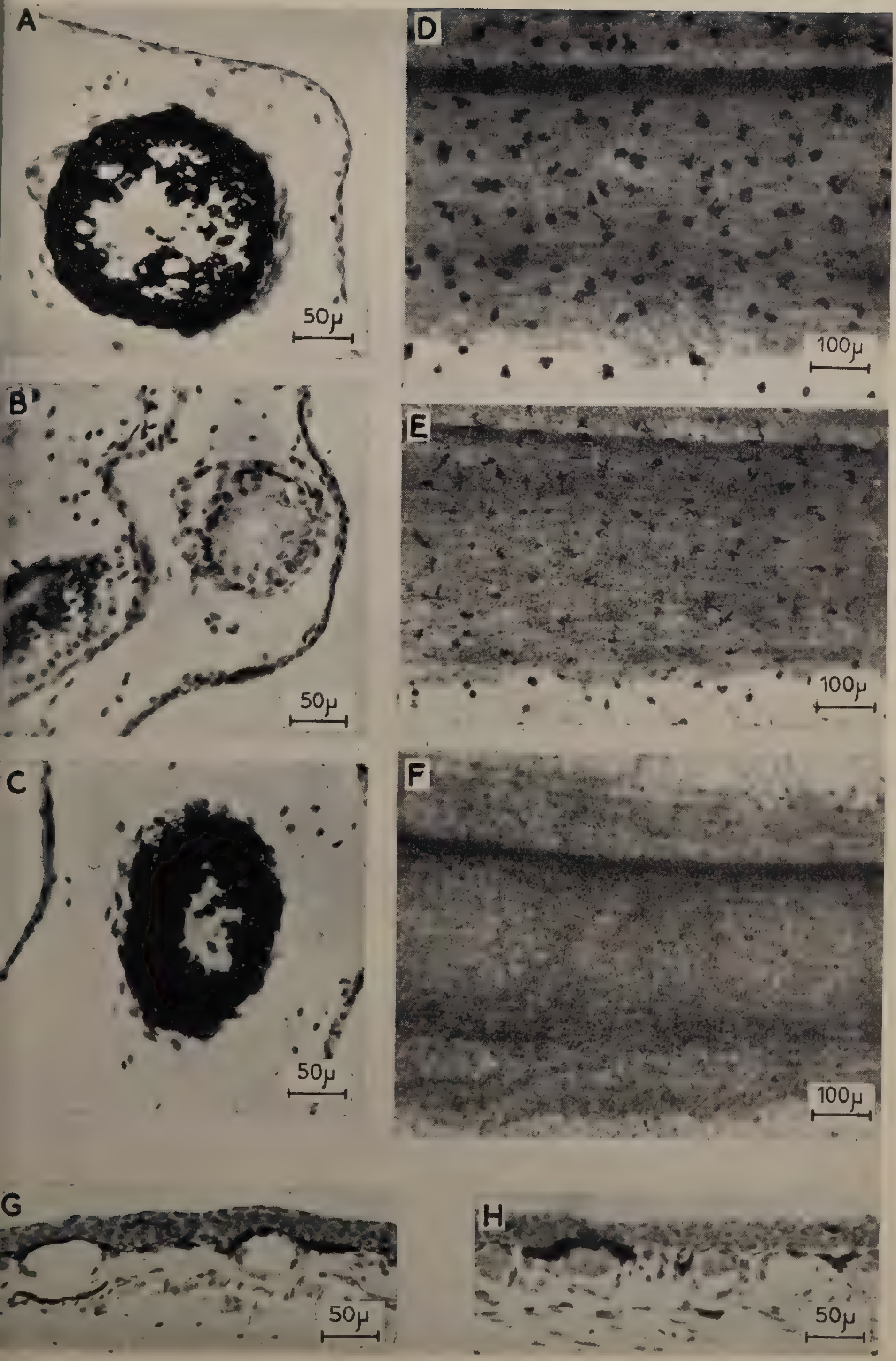

FIG. 2

R. T. SIMS

liver, along the walls of the sinuses. Occasional melanophores were insinuated about the bases of the epithelial cells of the intestine.

The pigment within the melanophores of the treated animals was a very light yellow in the whole-mount specimens. It was visible only in the meninges and on the superficial surface of the axial musculature; the pigmented coat of the eye was light yellow (fig. 2, B). Scattered throughout the liver were small areas of pigment varying in colour from light yellow to black. The coiled intestine was uniformly dark brown, with pitch-black areas distributed in its walls. Where it was present, the pigment in the melanophores of the sectioned material of the treated animals was of the same yellow colour as that seen in the whole mounts. The number of pigment granules in each cell was judged to be less than in the control series, as each granule was distinct from the others in its cell, while in the control series individual granules could not be discerned as a result of overlapping and super-positioning. The amount of pigment in each section was markedly less in the treated group than in the controls. This was especially noticeable in the meninges, axial musculature, and pronephros. The greatest amount of melanin present in the treated animals was in the liver and coiled intestine. As in the control series the pigmented cells were on the walls of the liver sinuses and even here the granules were separate in the treated animals in contrast to the controls. The coiled intestine was the only site where melanin was more abundant in the treated animals than in the controls. The melanophores were insinuated about the bases of cells of the intestinal epithelium and were full of black granules. No melanin was found in the region of the nasal pits, the otic capsules, the parietal peritoneum, scattered over the head immediately beneath the epidermis, in the lungs, or in the mesentery of the intestine. These were sites in which it developed in those animals that had their treatment stopped on the 9th day of the experiment. Apart from the marked difference in pigmentation, the structure of the eyes in the treated group differed in no respect from those of the controls.

Experiment 2. A large batch of tadpoles reared from the same spawn as those used in the first experiment was taken when they had reached stage 46 or 47, as at this point they had well-developed melanophores, feeding had begun, and the tadpoles were breathing air. A number were fixed and the rest were divided into two groups, one of which was reared in tap-water while the other was reared in a solution of phenylthiourea in tap-water. Six weeks later all the animals were staged and fixed.

The animals fixed at the beginning of the experiment were at stage 46 or 47. In sections their thyroid glands were seen to consist of large groups of cells; no follicles were present. The distribution of melanophores, which contained large amounts of black pigment, was identical with that found in the control group at the end of experiment 1.

The stages that the animals had reached by the end of the experiment are presented in the histogram (fig. 3). Almost nine-tenths of the control population were at stages 55, 56, or 57, whereas the treated group were still at stage

46 or 47. The thyroid glands of the control group were made up of large follicles with deeply pigmented melanophores in the connective tissue between them. The epithelial cells lining the follicles were about twice as high as they were broad and contained chromophobe vacuoles: the colloid also contained many chromophobe vacuoles. In the treated group the thyroid glands were made up of large follicles, surrounded by a cuboidal epithelium with scanty cytoplasm and no chromophobe vacuoles in the cells or the colloid. No melanin was present in the connective tissue.

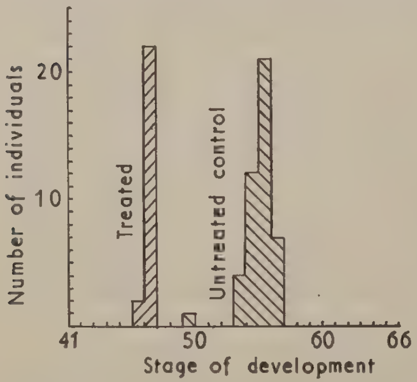

FIG. 3. *Experiment 2*. Histogram showing the number of *Xenopus* tadpoles at each stage of development. ▨ treated with phenylthiourea for 6 weeks; ▧ untreated control group.

The melanin of the experimental animals (fig. 2, C) was pitch-black and had exactly the same distribution as that of the normal animals fixed 6 weeks earlier. As the stages of the experimental animals were so different from those of the control animals at the end of the experiment, it was felt that a comparison of melanin in these two groups was unwarranted. A more reliable comparison was obtained by comparing the experimental animals at the end of the experiment with those fixed at the beginning, as they were at the same stage of development.

Experiment 3. 132 tadpoles, all at stage 49, were taken from a batch obtained from two different adults from those used in the first two experiments. They were kept in phenylthiourea solution for 12 days in order to overcome any latent period between the beginning of administration of the drug and the full effect of its action: during this time 7 of the tadpoles died. They were then all staged again, and without exception found to be still at stage 49. The last 5 mm was cut off their tails while they were lightly anaesthetized and a number of the amputated segments were fixed. Over the next 15 days the amputated portion of the tails regenerated and this was allowed to occur in tap-water with 40 of them and in phenylthiourea solution with the remainder. When the tails had regenerated, the two groups were examined for the presence of melanin in the regenerate of the living animals and a small number were fixed. No melanin could be seen in the treated group, while an abundance was present in the control group kept in tap-water. At this point half of the treated group were transferred to tap-water for 4 days to allow melanogenesis to begin. At the end of this time black melanin granules had just begun to appear and the treatment with phenylthiourea was started again. In this way 3 groups of experimental animals were obtained and designated A, B, and C. Group A had been kept continuously in tap-water since the tails had been amputated, group B had been kept continuously in phenylthiourea solution since the beginning of the experiment, and group C had been kept under the same conditions as group B except for 4 days, when it was allowed to form a small quantity of melanin. From the time of appearance of this melanin the experiment was allowed to run for 6 weeks; then all the surviving animals were staged, all the members of groups A and C and half the members of group B were fixed. The rest of group B had their treatment stopped and were kept in tap-water until melanin could be seen in the regenerates, when they were fixed.

The melanophores in the amputated segments of the tails were packed so full of black melanin that individual granules could not be distinguished. They were distributed in the dorsal and ventral fins, over the tail-muscles, and in the meninges. After regeneration had taken place the regenerates of those kept in tap-water contained melanophores in the same sites as the amputated segments, and again their colour was pitch-black and the individual granules could not be distinguished. On the contrary, in the treated group the number of melanophores containing melanin decreased rapidly at the base of the regenerate and so did the number of granules within them, until no melanin could be seen in the distal three-quarters of the regenerate.

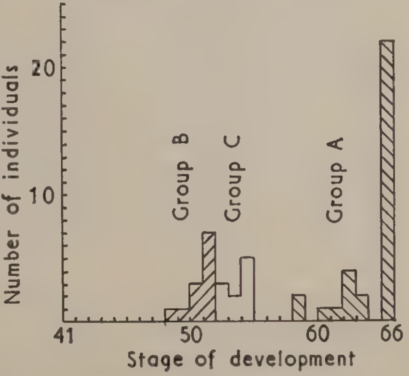

FIG. 4. *Experiment 3.* Histogram showing the number of *Xenopus* tadpoles at each stage of development. All the tadpoles were at stage 49 at the beginning of treatment and all had the last 5 mm of their tails amputated. ▧ group A treated for 12 days with phenylthiourea and then kept in tap-water for 8 weeks; ▨ group B treated continuously for 10 weeks with phenylthiourea; □ group C treated for 10 weeks with phenylthiourea, except during 4 days towards the beginning of the experiment.

It was at this point that the three groups were set up and group C was allowed to begin melanogenesis. The animals were examined every day for the appearance of melanin in the regenerate and it was noticed that it formed at the same time throughout this region and not in any one area before another. The distribution of the melanophores was the same as that in group A, kept in tap-water, but treatment was started again while the individual black melanin granules could still be distinguished and the melanophores were not packed full of them. There was no change in the regenerates of group B during this time.

The stages of development that the 3 groups had reached by the end of the experiment are presented as the histogram (fig. 4). It can be seen that group A were about 10 stages in advance of groups B and C, and group C was slightly ahead of group B. The thyroid glands of groups B and C consisted of large follicles, surrounded by cuboidal epithelial cells that were strongly basiphil. No chromophobe vacuoles were present in the colloid or in the cells. In group A the thyroid glands consisted of large follicles in which the epithelial cells formed a larger part of the follicle than did the colloid. The epithelial cells were columnar and had a long axis about 10 times greater than their transverse axis, and both cells and colloid contained numerous chromophobe vacuoles that were confluent in the latter.

At the end of the experiment the tails of group A were in various stages of regression. However, in the less mature members of the group the melanophores of the regenerates were distributed in the same manner as before and contained too much melanin for the individual granules to be discerned (fig. 2, D). In group B the melanin in the regenerates showed no change. The melanophores containing melanin decreased rapidly in number at the base of the regenerate as did the amount of melanin within them, and in the distal three-quarters of the regenerate melanin was absent (fig. 2, F). In the part of the tail not removed by the operation the melanophores were still so full of melanin that the individual granules could not be discerned. The regenerates in group C contained melanophores in which melanin was still present as before (fig. 2, E) except at the very tips where it was absent or present in only one or two cells.

Although the mortality was high, the deaths occurred uniformly throughout the experiment and did not warrant reducing the concentration of phenylthiourea or shortening the duration of the experiment. When dead animals were found they were fixed immediately and the regenerates examined for melanin. In all cases it was found that their appearance at death corresponded to that of the survivors in the group to which they belonged.

The members of group B that were kept in tap-water until they developed melanin all did so within 14 days, and it appeared at the same time throughout the regenerate and not in one place before another.

Experiment 4. Twenty-six tadpoles all at stage 66 were taken from a batch obtained from two different adults from those used in the first 3 experiments. At this stage only a small stump of a tail remains to be absorbed before metamorphosis is completed. Eighteen were kept in a solution of phenylthiourea in tap-water and 8 kept in tap-water as controls. The experiment was run for 6 weeks and then all the surviving animals were fixed: during this time 7 of the treated animals died. When the experiment was terminated all the animals in both control and treated groups had completed metamorphosis.

Macroscopic examination revealed only one difference between the two groups of animals. The claws of the 3 pre-axial digits of the foot were black in the control group and brown in the treated group. The thyroid gland with the larynx, the liver, and the right hind-limb were dissected out of each animal for sectioning.

The thyroid glands of the control group consisted of follicles that were only half full of stainable granular colloid, in which chromophobe vacuoles were infrequent. The epithelium was columnar, the long axis of the cells being twice as long as the width, and the nuclei were densely stained and compact. The thyroid glands of the treated group consisted of follicles that contained scanty stainable, homogeneous, hyaline colloid which contained numerous chromophobe vacuoles. The epithelium was columnar, the long axis of the cells being 5 to 10 times greater than their width, and the basal region of the cell was basiphil while the follicular region was eosinophil and granular. The nuclei were placed towards the free margin of the cells and were vesicular in appearance with prominent basiphil nucleoli.

In the livers and hind limbs of the control animals were many melanophores which contained an abundance of black melanin granules. As in the earlier stages those in the liver lined the sinuses. The melanophores of the hind limbs were distributed among the cells of the epidermis, immediately beneath the epidermis, along the blood-vessels, and in the marrow cavity of some of the bones, notably the phalanges. No difference in appearance or distribution was found in the treated animals (fig. 2, G–H).

The cornified claws of the control animals were pitch-black in sections whereas those of the treated group were light brown with a granular appearance, but neither group had any melanophores situated near this region of the epidermis or dermis.

Discussion

In the first experiment the formation of melanin was prevented by treating eggs with phenylthiourea. At the same time development of the treated group was slightly retarded but the histology of their thyroid glands was usual for the stage of development that they had reached. It is concluded that phenylthiourea at a concentration of 0·01% inhibits melanogenesis in tadpoles of *X. laevis* as it does in other Anura (Lynn, 1948; Millott and Lynn, 1954). Unlike the indophenol dyes used by Lewis (1932), no abnormalities of the optic cup were produced apart from the absence of melanin. This is evidence against her suggestion that melanin must be present in the pigmented layer if normal development of the eye is to occur. When tadpoles reared in the solution of phenylthiourea were transferred to water, melanin appeared simultaneously in the melanophores of all regions. This resembles the observations of Lehmann (1957) on *Amblystoma mexicanum* and supports the conclusion that lack of pigment does not influence the distribution of melanophores.

When it had been shown that phenylthiourea inhibited melanogenesis in eggs taken from one batch, the second experiment was undertaken to demonstrate the turnover of melanin. Tadpoles taken from the same batch were used. Thus genetic variations in the effect of phenylthiourea were minimized. Although the tadpoles were treated for 6 weeks, no loss of melanin was detected. This is in marked contrast to the disappearance of pigment in 5 days in *Eleutherodactylus* reported by Lynn (1948) and Millott and Lynn (1954). The check in development and the changes in the histology of the thyroid glands resemble those found by Gordon, Goldsmith, and Charipper (1945) in tadpoles of *R. pipiens* after prolonged administration of thiourea. This was regarded as evidence that phenylthiourea was reaching the tissues of the treated animals. This raised the possibility that phenylthiourea only prevented melanin formation if treatment was started immediately after the eggs were laid and not if treatment was started at later stages. The third experiment was designed to cover this eventuality.

The basis of the third experiment was that 3 groups of experimental animals, designated A, B, and C, were treated in different ways to influence the melanophores of tail-regenerates. They were all reared from a single batch of eggs and at the beginning of the experiment were all selected at the same stage of development. Group A were controls kept in tap-water. They showed the formation of melanin in the melanophores of the regenerate, the development of untreated tadpoles, and the histology of the thyroid gland. Group B were treated with phenylthiourea continuously for a period of 10 weeks. They showed the inhibition of melanogenesis in the regenerate and lack of turnover in the rest of the animal. The treatment of group C with phenylthiourea was suspended for 4 days to allow a small amount of melanin of known age to appear in the regenerate. As in the tadpoles of the first experiment which had their treatment stopped, melanin formed simultaneously in the melanophores throughout the regenerate, confirming again the conclusion of Lehmann (1957) that lack of pigment does not affect the distribution of melanophores. No detectable loss of this new melanin occurred during the following 6 weeks' treatment. The absence of melanin at the tip of the tail at the end of the experiment was regarded as evidence that inhibition of melanin formation occurred. The development of group C was arrested at a slightly later stage than that of group B, as a result of the short suspension of treatment. The histology of the thyroid glands at the end of this experiment was similar in groups B and C. The changes resembled those found in the second experiment, and indicated that phenylthiourea reached the tissues of the animals.

In view of the fact that *Eleutherodactylus* has no tadpole stage in its development, the possibility that the turnover of melanin was faster in the adult form than in the tadpole form remained to be excluded. A fourth experiment was performed with animals possessing only a small stump of tail at stage 66. Again no diminution of melanin was detected after a period of 6 weeks' treatment. The changes in the histology of the thyroid glands, which resembled those described by Joel, D'Angelo, and Charipper (1949) in adult *R. pipiens* treated with thiourea, indicated that phenylthiourea reached the tissues of the treated animals.

These experiments show that in *X. laevis* tadpoles treated with phenylthiourea there is no significant turnover of melanin, melanin formed before the beginning of treatment is not destroyed, development is arrested, and changes are produced in the histology of the thyroid gland resembling those produced by thiourea. Re-examination of all the sections prepared from treated and untreated animals used in this investigation revealed no instance in which melanin granules were situated within epithelial cells of the skin, as described and illustrated by Ehrmann (1885) for an unspecified frog. This is consistent with the lack of turnover of melanin in the treated animals. Once melanin has appeared it remains in the melanophores of this animal. They would be a sensitive indicator of the efficacy of inhibitors of melanogenesis during life, and make it easy to compare the action of these agents *in vitro* and *in vivo*.

I wish to thank Professor J. D. Boyd for his advice and encouragement during the course of this investigation, and Mr. J. F. Crane for the preparation of the photographs.

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Structure and Movements of Tick Spermatozoa (*Arachnida, Acari*)

By LORD ROTHSCHILD, F.R.S.

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With five plates (figs. 2 to 6)

SUMMARY

1. The spermatozoa of the tick, *Ornithodoros moubata*, which until now have been reported to move without any apparent means of propulsion, have been examined under the light and electron microscopes.

2. The surface of the spermatozoon is covered with ridges, running parallel to the long axis of the body, 0·30 to 0·47 μ long and 500 to 1,000 Å wide.

3. In longitudinal section approximately every other ridge exhibits wave-like deformations.

4. On the basis of these sub-microscopic structures and the behaviour of polystyrene balls stuck to the surface of live spermatozoa, the hypothesis is put forward that tick spermatozoa move by propagating lateral bending waves along the ridges in an antero-posterior direction.

5. No '9 + 2' or '9 + 9 + 2' arrangement of fibrils has been observed anywhere in tick spermatozoa.

6. There is a striking though not complete resemblance between the sub-microscopic surface structure of tick spermatozoa and that of gregarines.

INTRODUCTION

TICK spermatozoa are some of the most unusual in the animal kingdom. They may be just under a millimetre in length whereas the average spermatozoon, such as that of man or a sea-urchin, is about 60 μ long; during spermatogenesis, the head and acrosome are said to move from the 'front' to the 'back' end of the spermatozoon; ripe spermatozoa are only found in the female genital tract, where they are twice as long as in the male; the spermatozoa swim backwards (if the anterior end is defined as that where the head is located (fig. 1)); finally, the spermatozoa have, until now, been described as swimming or moving without any visible means of propulsion.

The general morphology of tick spermatozoa has been described by a number of scientists such as Christophers (1906, *Ornithodoros savignyi*, *Rhipicephalus annulatus*, and *Hyalomma aegyptium*); Casteel (1917, *Argas miniatus*); Nordenskiöld (1909, *Ixodes reduvius*); Oppermann (1935, *A. columbarum*); Tuzet and Millot (1937, various species of *Ixodes*); and Sharma (1944, *H. aegyptium*, *R. sanguineus*, and *A. persicus*). The spermatozoon is shaped like an Indian club (fig. 2, A, B), though the body is very flexible and often bent or twisted. The head is a long, thin structure inside the cytoplasm, at the posterior end. Mitochondrial granules are distributed throughout the cytoplasm, but they are especially dense at the anterior end, at the front of

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which there is said to be a ring-shaped centriole (fig. 2, B). Tick spermatogenesis has been described in detail by Sharma (1944), who also explained, as did Christophers in 1906, how ripe spermatozoa in the female genital tract come to be twice as long as those removed from the male.

The means by which tick spermatozoa move has puzzled previous investigators. Christophers (1906, p. 42) said they 'may exhibit a steady gliding movement, together with marked vermicular contortions of the anterior portion of their substance'. Sharma (1944, p. 313) said that 'The movement of the ripe spermatozoa takes place by the peculiar rotatory movement of the centrosome', but it is far from clear how a rotating centrosome or centriole could propel the spermatozoon. Nor have I seen this rotating centriole, though irregular, writhing movements of the anterior end can often be discerned, whether the spermatozoon is moving or not. Sharma also said that 'When the ripe spermatozoon is moving the mitochondria seem to be in a state of commotion. . . . It may be that these mitochondrial granules help in the peculiar movement of the spermatozoon.' It might be inferred from these statements that tick spermatozoa are neither ciliated nor flagellated and this is correct. (Sharma (1944) uses the word 'flagellum' to describe a long thin

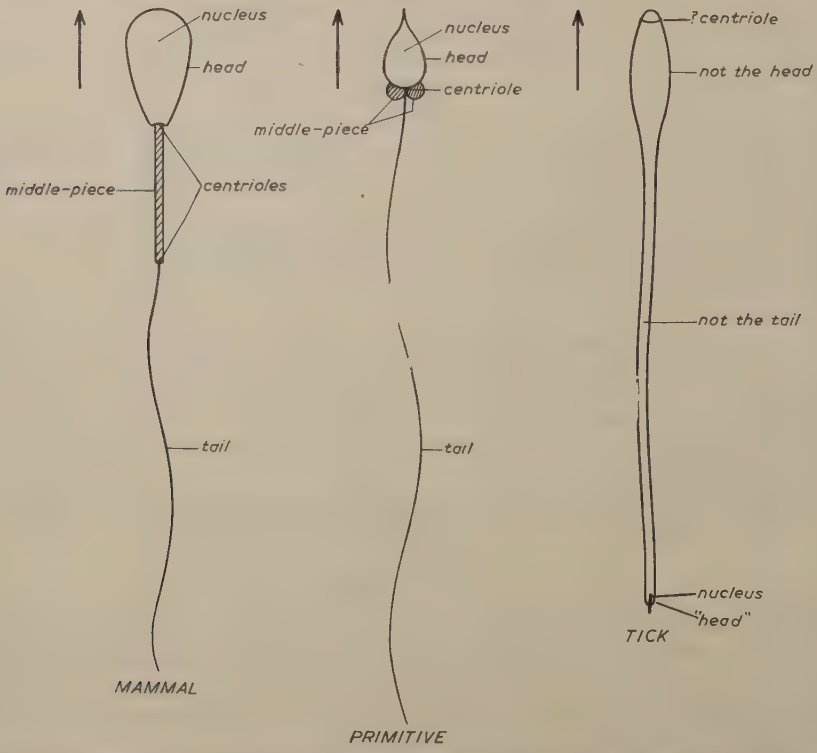

FIG. 1. Comparison of two common types of animal spermatozoa with that of the tick, *O. moubata*; not to scale. The arrows show the directions of motion.

structure in the spermatozoon of the fowl tick, *A. persicus*. The structure in question is not a flagellum in the normal sense of the word.) So far as I am aware, all spermatozoa which move do so either by the amoeboid method, like immature *Ascaris* spermatozoa (Panijel, 1951), or by propagating bending waves along their tails, which invariably contain the '$9 + 2$' or '$9 + 9 + 2$' system of fibrils. The body of a moving tick spermatozoon is not amoeboid and it does not bend; no '$9 + 2$' or '$9 + 9 + 2$' system has been seen; nor is there any evidence of 'jet propulsion' as in dragonfly nymphs (Hughes, 1958).

The object of the experiments described in this paper was to identify the structures concerned in tick sperm-movement and to try and find out how they work. Some progress is believed to have been made, though the structures themselves and their presumed mode of action are unusual.

MATERIAL AND METHODS

Spermatozoa of *O. moubata* Murray were obtained from spermatophores in female ticks. The spermatozoa were suspended in arachnid Ringer whose composition was 114 ml 67 mM KH_2PO_4, 144 ml 67 mM Na_2HPO_4, 347 ml 540 mM NaCl, 5·6 ml 360 mM $CaCl_2 . 6H_2O$, 5·6 ml 360 mM $MgCl_2 . 6H_2O$, 544·4 ml H_2O (compare Parry and Brown, 1959). The pH was 6·30 and the freezing-point depression Δ 0·67, which is the same as that of tick blood. A Zeiss 'planachromat' objective, ×100, with variable aperture, was used for examination by dark-ground illumination, with a mercury arc lamp.

For electron microscopy, the spermatozoa were fixed and prepared in the following ways: (1) 123 mM Na_2HPO_4, pH 7·4, Δ, 0·53, containing 1% OsO_4 for 1h; washed in 123 mM Na_2HPO_4, pH 7·4; alcohols; methacrylate (85% butyl, 15% methyl methacrylate); sections, 350–500 Å thick; grids floated on saturated solution of uranyl acetate in 50% alcohol for 2 h; washed in water. This way of preparing the material was unsatisfactory. (2) The same as (1), but with arachnid Ringer substituted for 123 mM Na_2HPO_4. This was even more unsatisfactory. (3) The same as (2), but with acetone-araldite substituted for alcohol-methacrylate. (4) By a method similar to that of Kellenberger, Sechaud, and Ryter (1959). The procedure was as follows: 3 ml of sperm suspension in arachnid Ringer containing 1% Bacto Tryptone (Difco) + 0·3 ml Michaelis acetate-veronal buffer, containing 1% OsO_4, pH 6·1, Δ, 0·67, 5 min; spermatozoa transferred to 1 ml acetate-veronal buffer, containing 1% OsO_4 + 0·1 ml arachnid Ringer + 1% BactoTryptone, pH 6·10, Δ, 0·67, and left overnight at room temperature; 8 ml acetate-veronal buffer added, 5 min; spermatozoa transferred to acetate-veronal buffer containing 0·5% uranyl acetate, 2 h; alcohols; methacrylate; sections. (5) The same as (4) but with acetone-araldite substituted for alcohol-methacrylate.

Sections were examined without removal of methacrylate or araldite on a Siemens-Elmiskop I electron microscope, with the assistance of Mr. R. W. Horne, the Cavendish Laboratory, Cambridge, and on a Philips 75-kV instrument.

RESULTS

Observations with the light microscope

Morphology. The general form of the spermatozoon is shown in fig. 2, A (dark ground) and B (phase contrast). According to Sharma (1944), a ring-shaped centriole can be seen in stained specimens at the anterior end. A rim can just be seen in fig. 2, B, in the right place. The site of this structure is invariably visible in sections (fig. 2, D), though it is not certain that the rim is a centriole. The cytoplasm at the anterior end is packed with granules (fig. 2, B), which, under the electron microscope, are seen to have a typical mitochondrial structure (fig. 4, A). Contrary to previous reports, these granules are not 'in a state of commotion' when the spermatozoon is moving. It is only when a spermatozoon has stopped moving and is moribund, as evidenced by subsequent disintegration, that intense Brownian movement of the granules is evident. The nucleus, which is at the distal end, cannot be clearly seen in fig. 2, A, B; the tail-like structure at the posterior end (fig. 2, A) is the 'head'.

Speed. The mean speed of 11 spermatozoa was 17·1 μsec^{-1}, standard error of mean, 4·1 μsec^{-1}, range 2·3 to 22·9 μsec^{-1}. The inclusion of 0·01 M ATP in the suspending medium had no effect on the movements of motile or motionless spermatozoa. This high concentration was deliberate, because the ATP was applied externally.

Tick spermatozoa sink to the bottom of a drop of arachnid Ringer. With normal methods of viewing, therefore, they move over a solid surface. They also move in a hanging drop, though they naturally sink to the bottom of it. These observations do not answer the question whether tick spermatozoa would move or swim in a medium whose density was such that they did not sink in it. Experiments to examine this point present difficulties and, for reasons discussed later, might not be of much help in elucidating the locomotory mechanism.

'Flagella'. When examined by dark-ground illumination, the spermatozoa were found to have fine filaments, as much as 10 μ long and reminiscent of bacterial flagella, on some parts of their surface. No reliable estimate of the diameter of the filaments could be made from examination by dark ground illumination, because, in such circumstances, there is no theoretical lower limit to the thickness of a line which can be detected, as there is in the case of direct illumination. (If structure a is wider than structure b, the amount of light scattered from a will be greater than from b and there will, therefore, be an increase in the amplitude of the diffraction pattern; but the width of the pattern may be unchanged.) Bearing these points in mind, the diameter of the

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FIG. 2 (plate). A, live tick spermatozoa; dark-ground illumination. The head is at the extreme 'posterior' end.

B, live tick spermatozoon; phase contrast.

C, surface of a tick spermatozoon, showing fibrillar structure; dark-ground illumination.

D, anterior end of tick spermatozoon in longitudinal section; araldite.

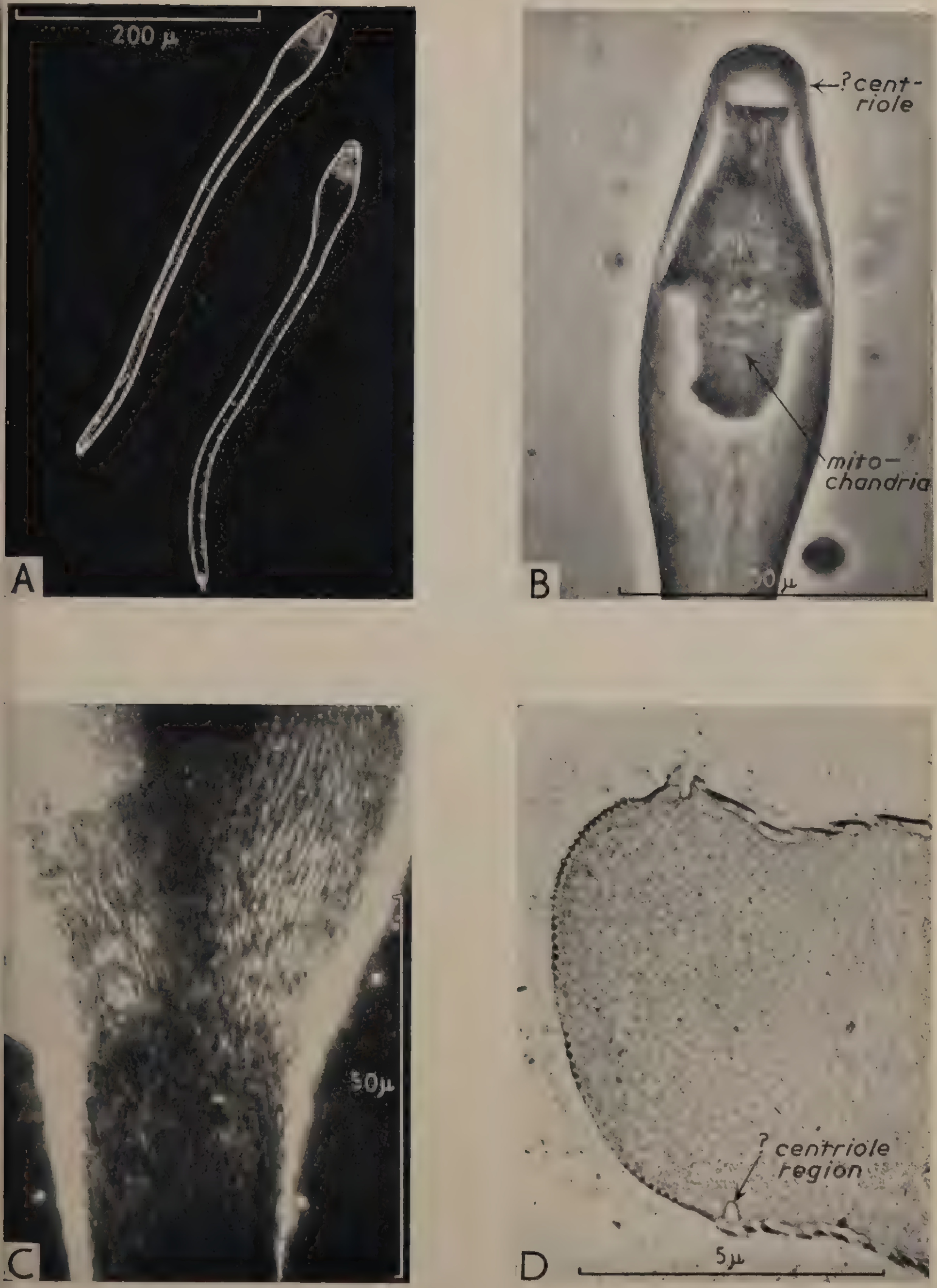

FIG. 2

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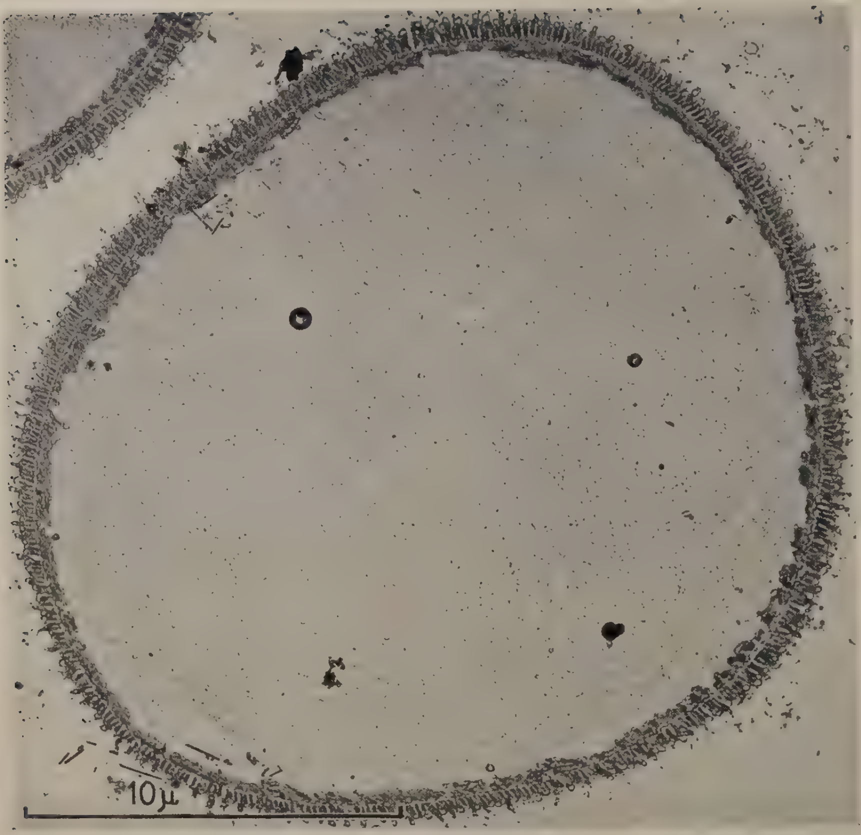

FIG. 3

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filaments was believed to be less than 5,000 Å when examined by dark-ground illumination. The filaments are invisible by phase contrast. Though theoretically possible, it was difficult to photograph the filaments because the amount of light they scattered was exceedingly small; moreover, they exhibited intense Brownian movement which precluded long exposures. The amount of light scattered during a few 30-μsec 50-joule flashes from a high-speed multiple flash-lamp (Brown and Popple, 1955) was insufficient to photograph them. The Brownian movement of the filaments could be reduced by suspending the spermatozoa in a viscous medium; but as a number of people in this Department have seen the filaments, little time was wasted in trying to photograph them, though one is visible on the original positive from which fig. 2, C was made.

The filaments have been seen on dead tick spermatozoa and on those which were not moving but which were believed to be alive. They were said above to be in a state of Brownian movement, because no cause for their movements other than thermal agitation is available in the case of dead spermatozoa. This does not, however, mean that the filaments move or vibrate in a random way. They assume a characteristic undulatory form, as if, because of anisotropic mechanical properties, they can only respond to Brownian forces in a particular geometrical way. No differences were discernible between the form of the undulations along filaments on live and dead spermatozoa.

Normally, the filaments seem to lie along the long axis of the body of the spermatozoon and, every now and then, to get out of this position and assume one in which their long axes are more or less at right angles to the surface of the spermatozoon, when they are visible if maximum magnification and dark-ground illumination are used. The distribution of visible filaments on the surface of the spermatozoon is unpredictable and there is no certainty that they will be seen on any particular spermatozoon. This suggests that normally, the filaments may be part of a membrane of continuous structure on the sperm surface which itself has a fibrous or filamentous appearance (fig. 2, C). The spermatozoon is, therefore, unlikely to be covered with separate 'bacterial flagella' and it follows that tick sperm-movement is not caused by bending waves in separate filaments, if that is how multiflagellate bacteria move. This system of propulsion is improbable for other reasons, discussed later. The fibrous membrane on the surface of the spermatozoon must be assumed to be so fragile that, occasionally, filamentous elements in it become detached except at their proximal ends.

Movements of spheres on the sperm surface. The surface of the spermatozoon is sticky, so that if polystyrene spheres, diameter 1·2 μ, are suspended in the medium, those which are free in the medium exhibit Brownian movement, whereas those on the sperm surface do not. When, however, a spermatozoon is motionless but alive (as evidenced by lack of Brownian movement of the mitochondrial granules), polystyrene spheres on the sperm surface can

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FIG. 3 (plate). Transverse section of tick spermatozoon; methacrylate.

often be seen being transported along the surface in an antero-posterior direction, though occasionally they move forward. The movement of the spheres is jerky but sometimes sufficiently dramatic to merit the description 'snapping backwards', as if some barrier to movement (mucus?) were suddenly overcome. The simplest hypothesis to explain the predominatingly backward movement of the polystyrene spheres and the forward movement of the spermatozoon is that deformations in some structure at the sperm surface pass from the front to the back end of the spermatozoon. This statement implies the existence of a contractile system at or associated with the sperm surface. A striated or ridged structure can be seen in light micrographs (fig. 2, C). There is little possibility of finding out any more about these structures with the light microscope, or of determining the plane of the (so far) hypothetical deformations relative to the axes of the spermatozoon.

Observations with the electron microscope

Transverse sections. Figs. 3 and 4 show transverse sections of a tick spermatozoon. The surface is covered with processes, hitherto undescribed, of different lengths, every fourth process usually being a long one. The average lengths of the shorter and longer types are 0·30 μ and 0·47 μ. The width of the processes is 500 to 1,000 Å, depending on the region of the spermatozoon in which the section is cut. This width is somewhat less in araldite-embedded specimens (fig. 4, B). Wave-like deformations of the processes are often observed. There is a membrane 80 Å wide at the surface of each process; it is composed of two electron-opaque layers, each 30 Å wide, with an electron-transparent layer, 20 Å wide, between them. A peculiar and ubiquitous feature of Kellenberger-methacrylate specimens is the presence of a vacuole at the end of almost each process (figs. 3; 4, A). These vacuoles are not always present in Kellenberger-araldite specimens (figs. 3; 4, A).

The processes do not appear to be continuous with the body of the spermatozoon, at the surface of which there is a plasma membrane. Presumably, the material round and at the bases of the processes is mucus. As already mentioned, the surface of the spermatozoon is sticky.

There is a dense layer of mitochondria immediately inside the body of the spermatozoon (figs. 3; 4, A), while sets of regularly spaced 'fibrils', 70 Å in diameter and 36 Å apart, run parallel to the long axis of the spermatozoon (figs. 4, A; 5).

Longitudinal sections. If a structure appears as a finger-like process in transverse section it may be a ridge, and this was found to be the case by examination of longitudinal sections (fig. 5, A, B). The ridges run roughly parallel with the long axis of the spermatozoon. The wavy shape of the ridges is very plain in longitudinal sections, which also reveal a difference between

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FIG. 4 (plate). A, transverse section of tick spermatozoa, showing ridges on surface; methacrylate. *m*, mitochondria; *f*, longitudinal fibrils in transverse section.

B, transverse section of tick spermatozoon, showing ridges on surface; araldite.

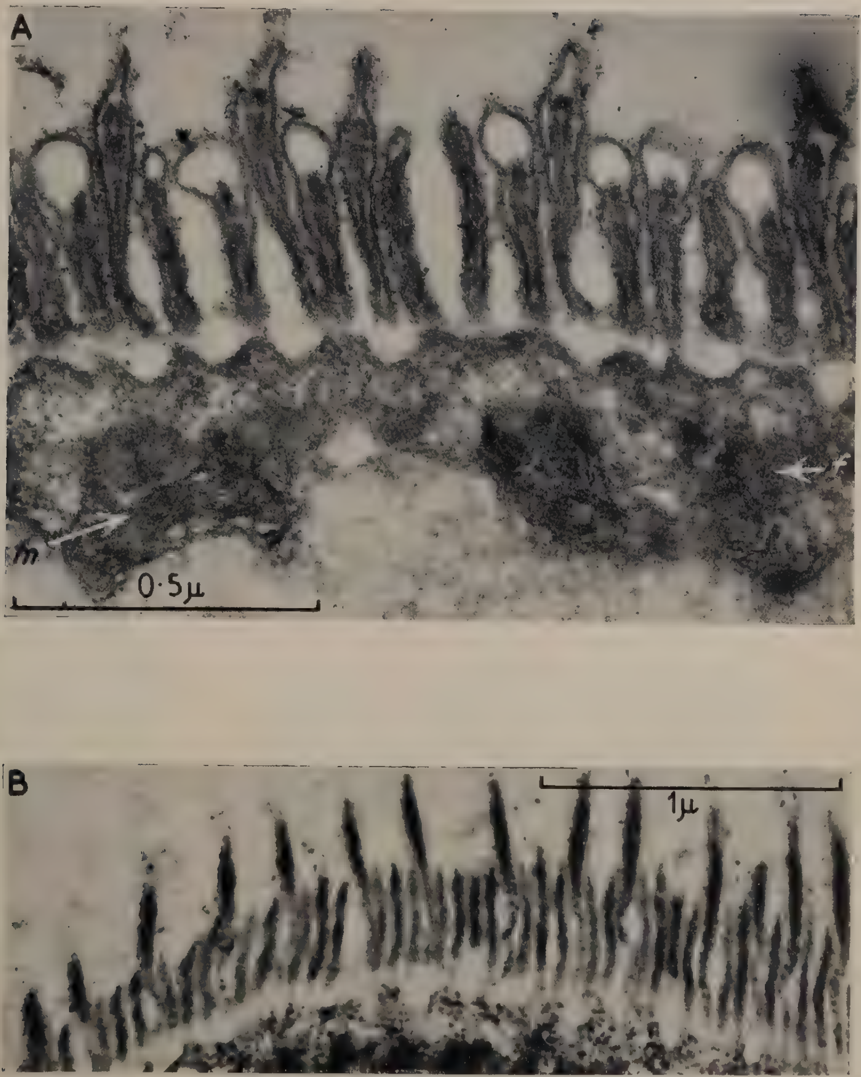

FIG. 4

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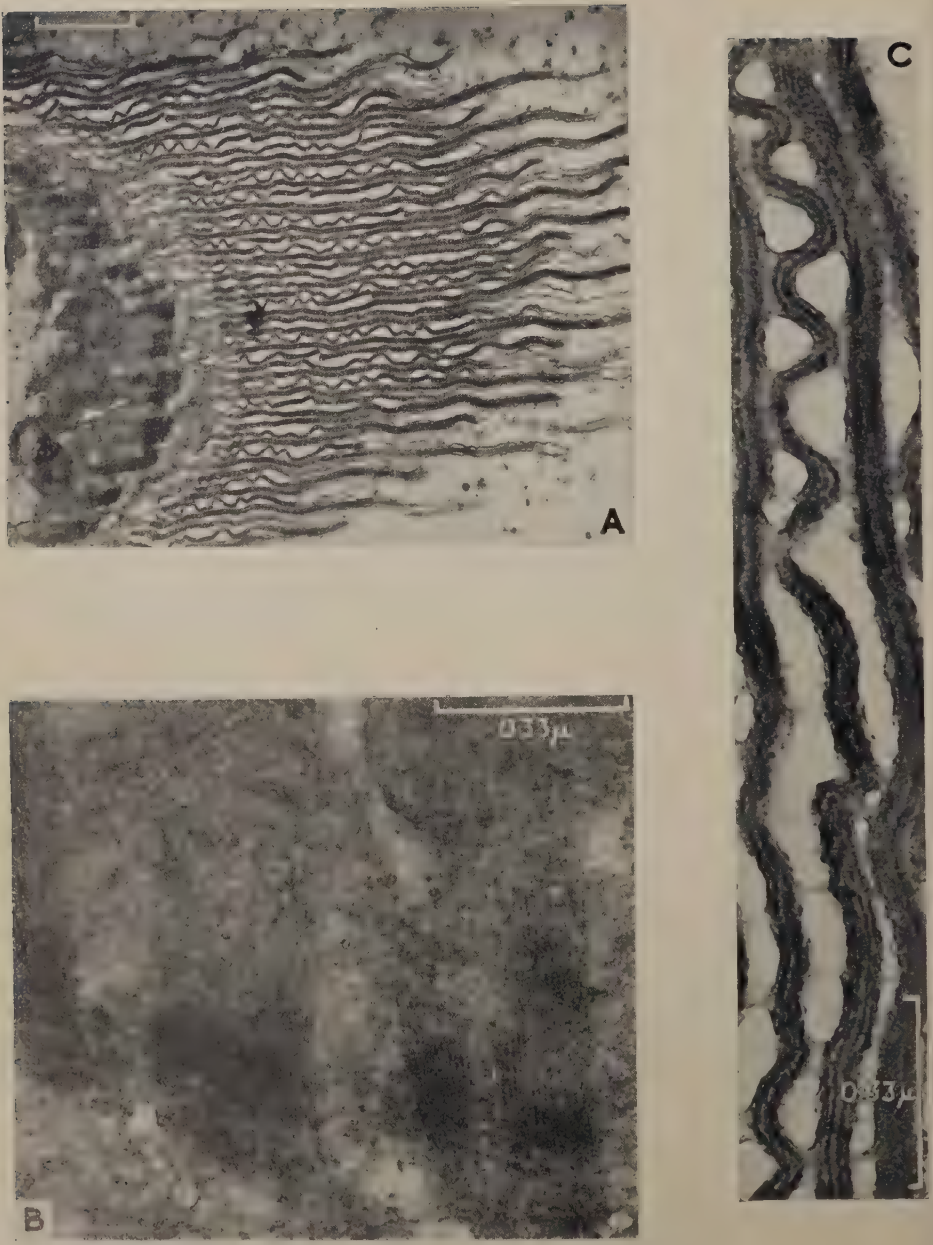

Fig. 5

LORD ROTHSCHILD

ridges, as some have waves in them whereas others do not. The periodicity of straight and wavy ridges is not completely regular, though there is an obvious tendency for a wavy ridge to have a straight one on each side of it. The ridges appear to have fibrils 80 Å in diameter within them. The word 'appear' is used because the fibrils were only visible when an 'unsatisfactory' fixative, e.g. 123 mM $Na_2HPO_4 + 1\ \%\ OsO_4$, was used.

Inexplicable structures. (*a*) In several sections believed to be in the region of the head, structures have been observed which cannot be identified with certainty (fig. 6, B). Outside the familiar finger-shaped processes there is a set of transverse sections of filamentous structures whose diameter varies from 650 to 1,200 Å. Some appear to contain sub-fibrils; others have cartwheel-shaped structures inside. As mentioned earlier, there are reasons for thinking that in certain circumstances, the ridges which run longitudinally along the body of the spermatozoon may get displaced from their normal positions. The fact that, at any rate for part of their length, they are not connected to the body of the spermatozoon (fig. 4, A), makes the possibility of their becoming detached more likely. The structures in fig. 6, A outside the finger-shaped processes may therefore be transverse sections of detached ridges. Their diameters are consistent with this possibility.

(*b*) In one, but only one, unshadowed whole preparation, 'flagella' were observed at one end (fig. 6, A). These are 4,300 Å in diameter. Although it would be convenient to ignore this electron micrograph on the grounds that it may be of some other organism which accidentally got into the preparation, I decided not to do this. Other shadowed and unshadowed whole preparations showed the ridges identified in transverse and longitudinal sections, though they were never displaced from the surface of the spermatozoon. The 'flagella' in this electron micrograph are not believed to be the same as those visible with the light microscope. The latter are probably displaced ridges.

Discussion

There seems little doubt that tick spermatozoa move by propagating bending waves along some, but possibly not all, of the processes revealed on their surfaces by the electron microscope. It has not so far been possible to decide whether deformations in these processes only propel the spermatozoon when it is in contact with a solid surface or whether they can do so when the spermatozoon is freely suspended in an aqueous medium. There is little hope of making further progress by observations on living tick spermatozoa because the processes are too small to be resolved with the light microscope.

Fig. 5 (plate). A, longitudinal section through ridges on surface of tick spermatozoon; methacrylate. This section is slightly oblique, the left-hand side passing through the body of the spermatozoon and the right-hand side through the ridges.

B, enlarged view of left-hand region of A, showing longitudinal fibrils seen in transverse section in Fig. 3, B.

C, part of a ridge in longitudinal section; methacrylate.

The irregular writhing movements which occur from time to time at the 'anterior' end of tick spermatozoa are unlikely to be connected with their translatory movements, though it is possible that the bending waves in the processes are initiated at the 'anterior' end, where mitochondria are particularly dense and where the processes may be connected to the body of the spermatozoon.

The processes bear a striking, but not complete, resemblance to those described by Kümmel (1958) in the gregarines *Gregarina polymorpha* and, particularly, *Beloides*; and it may, therefore, be that gregarines and tick spermatozoa move by the same mechanism.

The structure of the processes and the way they connect with the mitochondria underlying them will doubtless be further elucidated, if only because the electron micrographs reproduced in this paper are not entirely satisfactory. It is not at present certain, for example, whether the vacuoles regularly observed in specimens embedded in methacrylate are artifacts, or whether they reflect some secretory activity (the surface of a tick spermatozoon is very sticky). These vacuoles are not always seen in specimens embedded in araldite, but the Kellenberger method of fixation is believed to give as 'good' results with methacrylate as with araldite embedding. If more was known about the composition of tick body fluid or such liquid as there may be within the spermatophore, better results might be obtained.

As mentioned earlier, the 'flagella' observed with dark-ground illumination are unlikely to be separate organs of propulsion. It is far from clear how structures sticking out more or less at right angles to the surface of a spermatozoon and in a state of intense Brownian agitation could propel it forward, even if bending waves were propagated along them as is postulated in bacterial flagella. As Gray (1951) suggested in the latter case, the disorientated fibrils of tick spermatozoa are more likely to be part of a membranous sheath composed of a series of parallel fibrils which, under certain circumstances, become torn out of the sheath.

From what has been said above, it is evident that more work is needed to obtain a true picture of the structures responsible for tick sperm-movement; and the same applies in regard to the normal location of the fibrils which are occasionally seen in abnormal positions. But some progress has been made in the main objective of the experiments, to identify the organ of propulsion and suggest a method by which it works.

I am indebted to Sir James Gray, F.R.S., and Dr. A. V. Grimstone for commenting on the typescript of this paper. This work is supported by the Agricultural Research Council.

FIG. 6 (plate). A, unshadowed electron micrograph of one end of a tick spermatozoon, showing 'flagella'.

B, structures occasionally seen at anterior end of tick spermatozoon; methacrylate. For further details, see text.

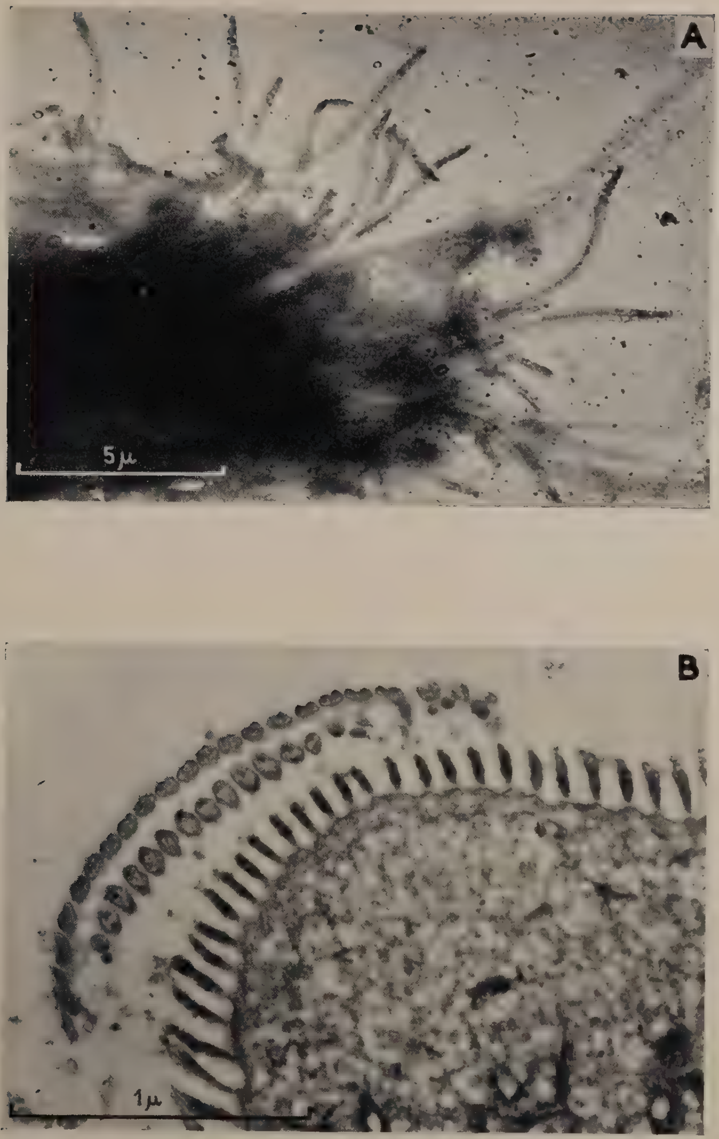

FIG. 6

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The Shell of Acridid Eggs

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SUMMARY

The shell of the newly-laid egg of *Locusta migratoria* is composed of a chorion and an outer layer, here termed the extrachorion. The development of the chorion and extrachorion has been followed. The structures of the egg-shells of *Schistocerca gregaria, Romalea microptera,* and 6 species of British grasshoppers are also described and are found to conform to the same pattern. The chorion has a meshwork structure which may be filled with air or water. Wound repair in the eggs is also noted.

INTRODUCTION

SLIFER (1937) mentions a temporary coating on the eggs of *Melanoplus differentialis*, a layer which Roonwal (1954*a*) terms exochorion. Jahn (1935) considers the chemical nature of the egg-membranes and shows a difference between the inner and outer layers of the chorion. An investigation of the egg-shells of some Acrididae revealed that the egg-wall in the freshly-laid eggs was not wholly of follicular origin and that it therefore should not be termed chorion. In this paper the chorion and extrachorionic layers are described in various Acrididae. Descriptions are given of the egg-shells of *M. differentialis* (Slifer, 1937, 1949), *Locusta migratoria* (Roonwal, 1954*a*), and *Schistocerca gregaria* (Roonwal, 1954*b*). Kulagin (1932) has also described the structure of the egg-shell of *L. migratoria* and *S. peregrina* (*gregaria*), but in *Locusta* he calls the chorionic layers 'ectochorion', the outer zone of the white cuticle 'endochorion', and the inner zone 'oolemma'.

THE STRUCTURE OF THE EGG-WALL OF *LOCUSTA MIGRATORIA MIGRATORIOIDES*

The egg of *Locusta* is about 6·5 mm long and 1·3 mm wide. The surface is covered by a series of granules except at the posterior end beyond the ring of micropyles, where there is slight hexagonal sculpturing. The wall of the freshly-laid egg consists of 4 layers in the following order, beginning with the innermost: (1) the viteline membrane, with the primary wax layer; (2) the endochorion; (3) the exochorion; (4) a layer which is here called the extrachorion.

The endochorion (fig. 1, *end*) is a meshwork layer, 4 to 8 μ thick, except at the posterior end where it is up to 30 μ thick. This layer consists of an interlocking, tangled system of fine struts. Air in the spaces can be rapidly displaced by water or alcohol, which can enter through the inner surface or through the exochorion. Entry of water is even more rapid through cut or torn edges.

The exochorion (fig. 1, *exo*) is an outer layer, less than 1 μ thick, overlying

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the endochorion. Both the endo- and exochorion stain weakly with haematoxylin, strongly with eosin, and red with the Azan stain.

The extrachorion (fig. 1, *ext*) is a thin layer containing granules. The granules are 6 to 8 μ high and about 4 μ wide, but the remainder of the layer is very thin and is usually rather fragmentary. The nature and appearance of this layer changes with age as will be described in the following section. In the dried-out condition this layer stains strongly with haematoxylin and blue with Azan. The extrachorion has been called the temporary coating by Slifer (1937, 1949), but Roonwal (1954*a*) has termed it the exochorion. As this layer appears after the egg has separated from the follicular cells it can hardly be termed chorion. It is also rather more persistent in *Locusta* than it appears to be in *Melanoplus* (Slifer, 1937).

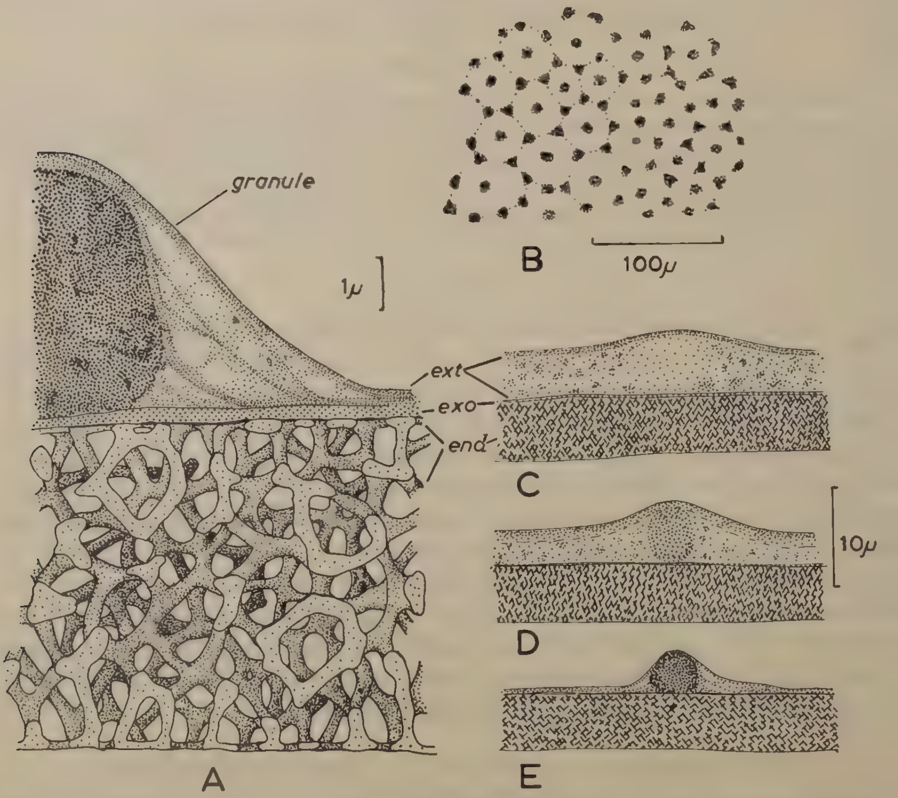

FIG. 1. Egg-wall of *L. migratoria*. A. the egg-wall 1 day after laying, showing part of a granule; B, surface view of chorion 1 hour after laying; the dots between granules on the left have been added to emphasize the hexagonal pattern; C to E, stages in the shrinkage of the extrachorion while the egg is in the common oviduct, showing development of a granule. *ext*, extrachorion: *exo*, exochorion, *end*, endochorion.

STAGES IN THE FORMATION OF THE EGG-WALL OF *LOCUSTA*

1. When the oocyte is fully formed the endochorion is secreted by the follicle cells.

2. The exochorion is secreted as a thin layer over the endochorion. It is without surface structure but when stained with haematoxylin it shows, in surface view, an indistinct hexagonal pattern. Each hexagon has an indistinct central spot. The hexagonal pattern corresponds with the boundaries of the follicle cells. It is not apparent in sections.

3. As the egg enters the common oviduct the extrachorion is secreted. It rapidly attains its maximum thickness and is fully present on any part of the egg by the time that part has passed 0·7 to 0·8 mm into the oviduct.

4. The extrachorion starts to shrink or condense while in the common oviduct. As the layer shrinks small islands remain. At first these islands are less granular than the rest of the layer (fig. 1, C). They correspond in position to the angles of the hexagonal pattern and to the central spots (see 2, above). As the extrachorion further contracts, the islands become denser while the rest of the layer shrinks down on to the chorion (fig. 1, D).

5. When the egg is laid the extrachorion is still not fully shrunk (fig. 1, E). The chorion is still filled with liquid. If exposed to the air it may dry out in a few hours. Under moist conditions, such as might be found in the egg-pod in damp soil, this may never happen. If the chorion dries out, the extrachorion also rapidly dries out into a thin layer, but under moist conditions the extrachorion will still continue to shrink, although more slowly. The denser islands of the extrachorion persist as the surface granules, and the rest of the layer remains as thin rather fragmentary sheets.

6. As the egg develops it absorbs water and expands. At first the chorion stretches, but later it may break. By this time, however, the serosal cuticle is fully formed and is the principal wall of the egg.

THE EGG-WALL OF OTHER ACRIDIDAE

Schistocerca gregaria. The egg is about 7 mm long and 1·3 mm wide. Its appearance has been described by Roonwal (1954*b*). The chorion has raised ridges forming an approximately hexagonal pattern corresponding to the edges of the follicular cells. The chorion does not show any differentiation into exo- and endochorion, although the outer zones are slightly more dense. The structure of the chorion, including the ridges, is similar to the endochorion of *Locusta.* There is no separation between the ridges and the endochorion as shown by Roonwal (1954*b*). A layer of extrachorion covers the egg, bridging over the ridges and leaving spaces beneath them. When the extrachorion is first produced it fills the spaces between the ridges. It later shrinks, while still in the oviduct, to form the layer over the ridges, and it also forms thickenings on the tops of the ridges, especially at the angles of the ridges.

Romalea microptera. The egg is 9 to 10 mm long and 2 mm wide. The structure of the shell is similar to that of *Schistocerca.* The raised ridges of the chorion form a deep honeycomb pattern which is bridged over by a layer of extrachorion. The chorion is about 30 μ thick and the ridges extend a further 100 μ outwards. The chorion shows two zones: the endochorion, which has a similar structure to that of *Locusta* and extends a little way up the ridges; and the exochorion, which forms a layer up to 8 μ thick over this. The structure of the exochorion is similar to the endochorion but the meshwork is much finer and denser. The extrachorion is present as a thin layer over the ridges and it also forms the thickenings on the tops of the ridges.

Chorthippus parallelus. The egg (fig. 2, A) is about 3·7 mm long and 1 mm wide. The external appearance of this and other eggs of the British Acrididae is described by Waloff (1950). It has a prominent sculpturing of low hexagonal ridges on the surface. The total thickness of the shell varies from about 35 μ at the sides to about 100 μ at the posterior end. The endochorion, which accounts for most of the thickness, has two distinct zones (fig. 2, B, C). The inner zone has a much finer and denser tangled meshwork than the outer zone. There is a thin, continuous exochorion about 0·5 μ wide and a

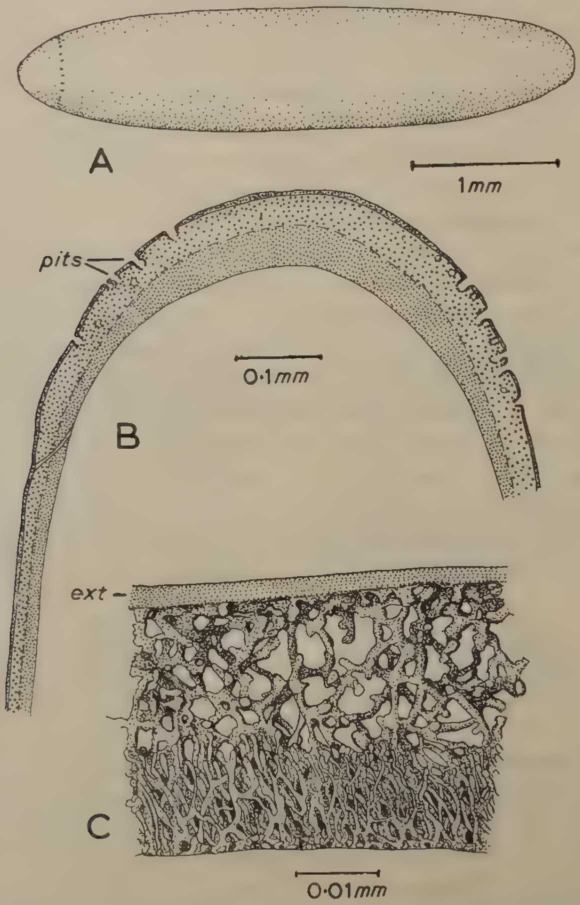

FIG. 2. Egg of *C. parallelus.* A, the whole egg. B, semi-diagrammatic section of the posterior end of the egg, showing pits, micropyle, extrachorion, and two zones of endochorion. C, the egg-wall of the side of the egg near the posterior end; note thick extrachorion (*ext*).

thicker extrachorion about 2 μ. There is a series of small pits forming a ring around the posterior tip of the egg. These pits pass through the outer layers and enter the outer zone of the endochorion. Water readily enters the chorion through these pits, but elsewhere the surface is hydrofuge.

Chorthippus brunneus. The egg is slightly larger than that of *C. parallelus* and is without sculpturing. The endochorion is about 8 μ thick and consists of a dense meshwork throughout. The appearance is very like that of *Locusta*, which is slightly less dense than the denser part of the chorion of *C. parallelus*. There is a slight tendency for the inner part to be denser. The endochorion is thicker at the posterior end. The exochorion and extrachorion are very like those of *C. parallelus*. The posterior end of the shell also has pits in it running into the endochorion, but these do not have any special permeability to water. The whole of the egg-wall takes up water fairly readily.

C. albomarginatus (*elegans*), *Omocestus viridulus*, *Stenobothrus lineatus*, and *Myrmeleotettix maculatus* all have an egg-wall structure very like that of *C. brunneus*. Their behaviour to water is also the same. The illustration of the egg-wall of *Melanoplus differentialis* (Slifer, 1949, fig. 7) indicates a structure similar in general form but with a thicker exochorion.

Discussion

In the newly-laid egg the layers of the shell are of maternal origin. During subsequent development a thick serosal cuticle, which is almost completely reabsorbed before hatching, is produced beneath the shell (Slifer, 1937). Jahn (1935) distinguished two layers in the chorion of *Melanoplus* eggs by their chemical reactions. Slifer (1949) refers to a 'mucus-like temporary coating' which covers the newly-laid egg. It is now found that the egg-wall is formed in two stages: (1) the chorion, which may itself be separated into two zones, and (2) an outer layer produced as the egg enters the oviduct. This outer layer is here called the extrachorion.

Kulagin (1932) claims that in *Locusta* possibly his ectochorion and also his endochorion are formed after the egg has left the ovary, and that the only layer present in the ovary is the oolemma. However, his fig. 4 clearly shows that both his endochorion and oolemma are parts of the serosal cuticle.

Slifer (1937) states that the temporary coating of *Melanoplus* eggs shrinks and disappears during the first few days after laying. This has not been found in the eggs examined. The extrachorion shrinks and may, as a result, crack; but it persists as a thin rather fragmentary layer. Further evidence of this persistence is shown in *Locusta*, where the surface granules are part of this layer. The granules are still evident in hatched eggs. During the process of expansion by the egg, all the egg-wall layers appear to become thinner as they stretch before breaking. This process has made some authors suggest that dissolution of the white cuticle starts at a much earlier stage than it does. The extrachorion in *C. parallelus* eggs renders the egg impermeable to water except at the posterior end. It is probable that this layer also has the same properties in *Locusta*, as the egg is not uniformly wettable. The layer in the latter is very thin and has many cracks. In other species of *Chorthippus*, which have an extrachorion nearly as thick as that of *C. parallelus*, no barrier to water absorption is produced, and in these eggs water enters anywhere through the surface. Water will enter a damp egg more rapidly than a dry one. The reason is that the chorion itself, rather than the air spaces, absorbs water and expands as a result. This in turn facilitates the entry of water into the spaces of the chorion.

The endochorion is composed of a close network of fine struts which produce a felt-like structure. This structure has many advantages for eggs of this nature. It conducts water, thereby enabling the egg to collect water efficiently through only a small area from a water supply anywhere on the surface of the shell. Under dry conditions water loss from the egg in the gaseous phase is restricted. The endochorion can also be compressed and stretched before it breaks, particularly when it is wet. This allows the egg to expand without destroying the means of water conduction or affecting the structural support. The chorion of a fully expanded egg cracks if it is allowed to dry, but at this stage the serosal cuticle has taken over the structural support of the egg. The frothy nature of the egg-pod also allows the eggs to expand while still allowing water and air to reach them.

The expansion of the egg involves a certain risk from abrasion, particularly if the eggs are laid in a pod in the ground. Jones (1958) shows that the liquid filling the space between the serosa and serosal cuticle can rapidly form a tanned protein-lipid membrane. This occurs when the liquid is released through punctures in the egg-wall. Slight damage by abrasion, and even by the effects of differential drying in strong light, cause small drops of this liquid to appear in swollen eggs. These drops immediately form a skin and then dry up. Examination of the swollen eggs of *Locusta* has shown that damage has occurred and been repaired in this way many times. The fluid in other acridid eggs also has this wound-repairing property.

I am grateful to Dr. H. E. Hinton for help and advice, and to Dr. B. P. Uvarov and the Anti-Locust Research Centre for the supply of *Schistocerca gregaria* and eggs of *Romalea microptera*, and of an English abstract of Kulagin (1932).

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The Development of the Polychaete *Haploscoloplos fragilis*

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SUMMARY

The eggs of *Haploscoloplos fragilis*, laid in gelatinous cocoons, develop as yolky trochophores which add 4 segments before hatching on the 6th day and two more during a pelagic phase before settling and the onset of feeding on the 10th day. The 6 (embryonic) segments bear a metatroch and 5 amphitrochs. The embryo also has an akrotroch, prototroch, telotroch, and neurotroch. All cilia disappear after settling, when the embryo shows resumption of segment formation. Anterior to the first trunk segment lie the prostomium and mouth region. The latter becomes subdivided by an annulus, but later fuses with the first trunk segment as the peristomium. All other segments become chaetigerous, the 5 embryonic being of thoracic type, the post-embryonic abdominal. At the posterior end of the body lies the pygidium, with the segment-forming growth zone immediately in front of it.

The development of trunk segments in two series, embryonic and post-embryonic, is characteristic of ariciids, as is the fusion of the first trunk segment with the mouth region to form the peristomium. It is possible that the mouth region incorporates at least one other cephalized segment.

Ariciid eggs vary in size among species. No direct relationship exists between egg size and pelagic development. The mode of oviposition also varies. Whether or not a pelagic phase occurs depends on the stage at which the embryo becomes free in the water, which is governed in turn by properties of the cocoon (e.g. jelly viscosity). A pelagic phase is always associated with enhanced ciliation, while in non-pelagic development ciliation is reduced. The basic ciliation (akrotroch, prototroch, metatroch, telotroch, and neurotroch) is usually supplemented by additional segmental bands.

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INTRODUCTION

THE external features of development have been described for several species of ariciid polychaete, but little attempt has been made to assess the general pattern of development and its variations in the family. The present study of the development of *Haploscoloplos fragilis* Verril and its comparison with the development of other species (*H. bustoris* (Horn &

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Bookhout, 1950), *H. kerguelensis* (Okuda, 1946), *Scoloplos armiger* (Anderson, 1959), *Aricia foetida* (Salensky, 1883; Lo Bianco, 1899; Schaxel, 1912) and *Nainereis laevigata* (Okuda, 1946)) have brought to light certain hitherto undisclosed features of polychaete life histories.

MATERIAL AND METHODS

H. fragilis is common in sandy intertidal flats at Botany Bay on the New South Wales coast (Anderson, 1960). Eggs are laid in the late winter (July and August) in cylindrical gelatinous cocoons some 10 cm long and 3 to 4 mm broad, one end of the cocoon being anchored in the sand. A layer of sand-grains adheres to the surface of the cocoon. Embedded in the jelly are several thousand eggs in spirally wound longitudinal rows. The early phases of development are passed in the cocoon. Hatching occurs at 6 days and a brief post-hatching pelagic phase is followed by settling and burrowing.

Cocoons were maintained in bowls of sea-water at 17° C. After hatching, the embryos were provided with beach-sand in which they burrowed after settling, feeding on diatoms and other organic material. Drawings of living embryos were made with the aid of a camera lucida. Phase-contrast microscopy was used to elucidate ciliation.

EXTERNAL FEATURES OF DEVELOPMENT

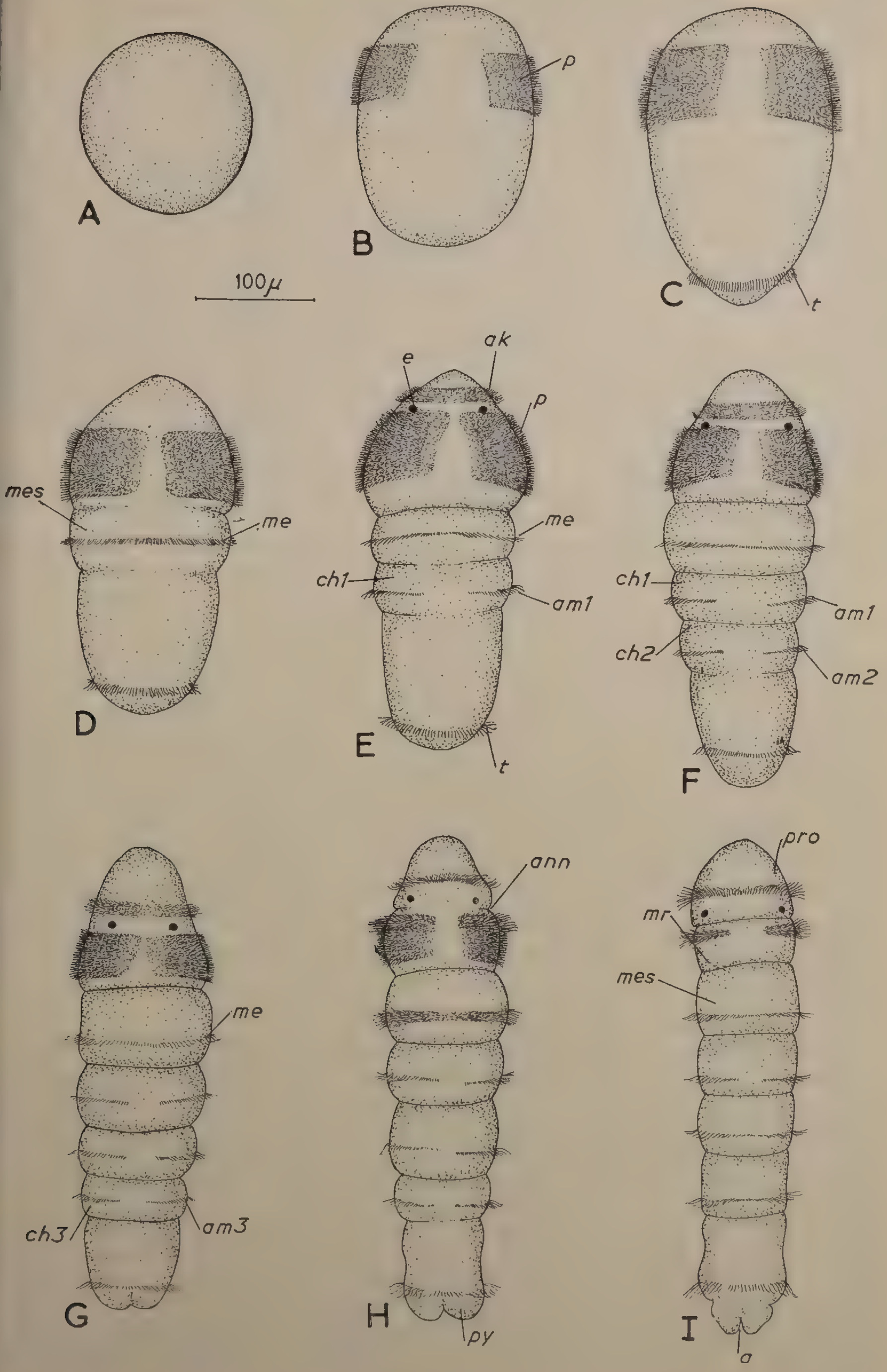
The embryonic period

The unfertilized egg of *H. fragilis* (fig. 1, A) is opaque, spherical, 150 μ in diameter, greenish brown to orange by reflected light, brown by transmitted light, and covered by a transparent egg membrane 2 μ thick. At fertilization the membrane becomes raised from the egg surface. After spiral cleavage and gastrulation it forms the embryonic cuticle.

Early on the 2nd day, prototrochal cilia (*p*) appear as a broad band on each side of the anterior half (fig. 1, B) and the embryo begins to elongate. Towards the end of the same day a telotroch (*t*) arises as a narrow band of longer cilia near the posterior end (fig. 1, C). The increase in length of the embryo results from activity of a growth zone in front of the telotroch. Behind the telotroch lies a terminal post-segmental pygidium.

During the 3rd day (fig. 1, D) an anterior prototrochal region is demarcated by an annulus from a narrow trunk, and at the anterior end of the trunk a pair of lateral grooves marks the posterior border of a metatrochal or first trunk segment (*mes*) on which a further circlet of cilia, the metatroch (*me*), is formed. The embryo does not normally move through the jelly of the cocoon

FIG. 1. A, unfertilized egg. B, early 2-day embryo, dorsal view. C, later 2-day embryo, dorsal view. D, early 3-day embryo, dorsal view. E, early 4-day embryo, dorsal view. F, early 5-day embryo, dorsal view. G, early 6-day embryo, dorsal view. H, early 7-day embryo, dorsal view. I, early 8-day embryo, dorsal view. *a*, anus; *ak*, akrotroch; *am 1*, *am 2*, *am 3*, amphitrochs of chaetigers 1, 2 and 3; *ann*, post-prostomial annulus; *ch 1*, *ch 2*, *ch 3*, chaetiger 1, 2, 3; *e*, eye; *me*, metatroch; *mes*, metatrochal segment; *mr*, mouth region; *p*, prototroch; *pro*, prostomium; *py*, pygidium; *t*, telotroch.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G
ann
H
py
pro
mr
mes
I
a

FIG. 1

at this stage, but if artificially released from the cocoon is able to glide by ciliary action through the water.

On the 4th day, as the trunk continues to grow (fig. 3, A, p. 264) the annulus at the posterior margin of the metatrochal segment is completed and a further pair of lateral grooves appears at the boundary of the second trunk segment (*ch 1*, fig. 1, E). An amphitroch, a pair of narrow half-circles of cilia, develops rapidly on this segment. At the same time the ciliation of the embryo is augmented by an akrotroch (*ak*), a circlet of cilia in front of the prototroch, and a neurotroch (*n*, fig. 2, A), a narrow mid-ventral band of cilia extending from the anterior border of the metatrochal segment to the telotroch. Slow ciliary gliding through the jelly of the cocoon now begins, and if released into the water, the embryo glides rapidly. Progress in the cocoon is greatly impeded by the viscous jelly. It is probably significant that the viscosity of the jelly lessens, so that the cocoon gradually liquifies, towards the time of hatching (see discussion, p. 271).

Muscular movements of the embryo also begin on the 4th day, with irregular elongation and contraction and lateral bending as far back as the anterior margin of the second trunk segment. A pair of dark-brown eye-spots (*e*) appears at the same time at the antero-lateral margins of the prototroch.

On the 5th and 6th days (fig. 1, F, G) two further trunk segments (*ch 2*, *ch 3*) are successively delimited. Each rapidly gains an amphitroch and takes part in the slow elongation-contraction movements of the embryo. On the 6th day the pre-pygidial growth zone becomes dormant (fig. 3, A), growth temporarily ceases, and the embryo hatches from the cocoon. During the next 4 days, as the tissues of the embryo differentiate towards their functional condition, no further segments are added to the trunk (figs. 1, G, H; 2, A; 3, A).

Just before hatching, several rows of cilia at the anterior end of the prototroch increase in length (fig. 1, H), while the prototroch as a whole lessens in area. A pair of lateral grooves appearing between the anterior border of the prototroch and the akrotroch also marks off the eye-bearing prostomium from the mouth region (*mr*), the latter including the prototroch and the ventral mouth.

On escaping from the jelly, the embryo (fig. 1, H) moves for a short time by ciliary gliding on the substratum close to the cocoon. It then becomes negatively geotropic and swims by means of its cilia towards the water surface. This behaviour persists for one to several hours. No photic response is involved, although the eyes are well developed, since no orientation towards a uni-directional light source is detectable in the pelagic embryo. In swimming,

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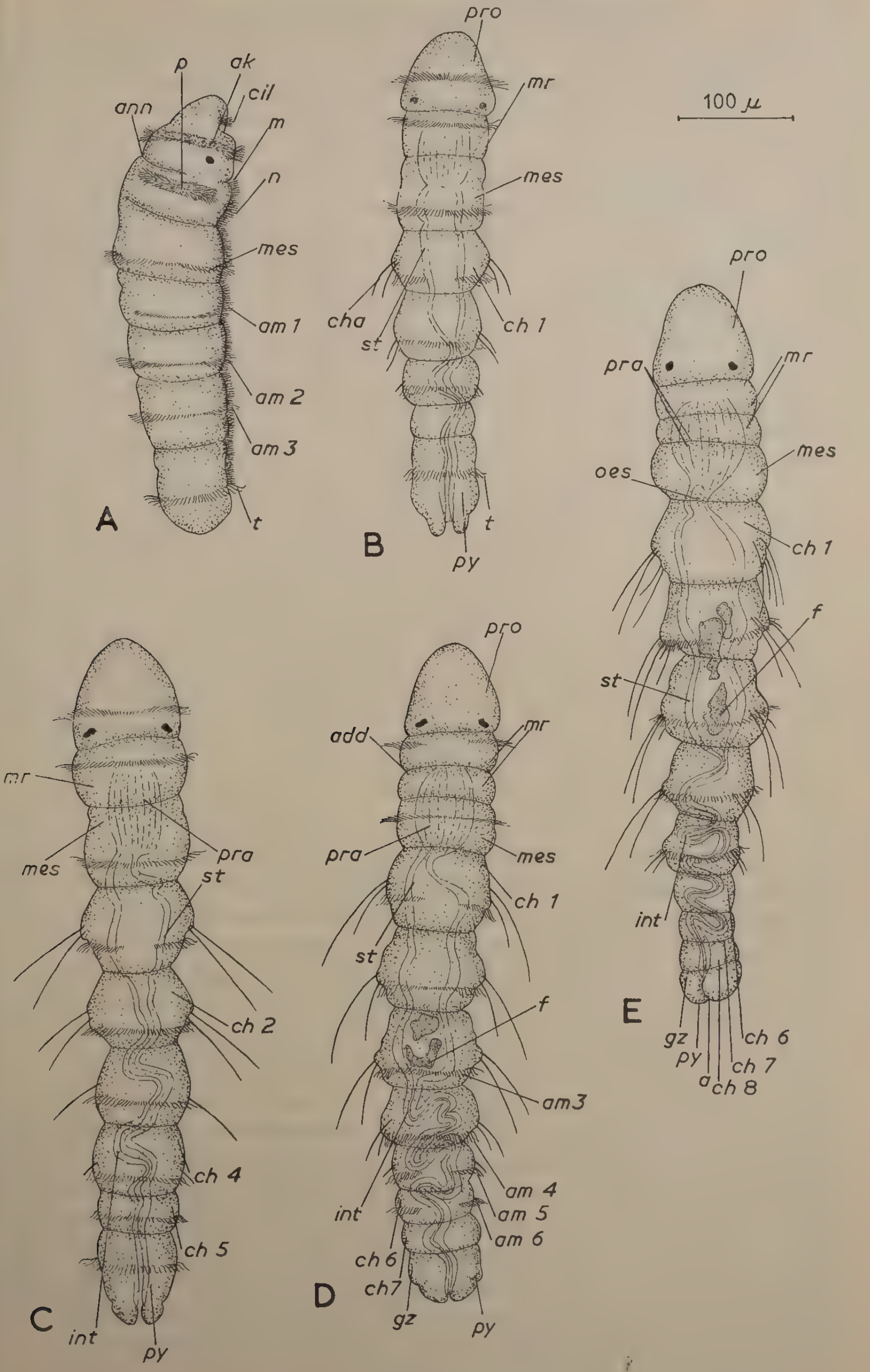
FIG. 2. A, early 8-day embryo, lateral view. B, early 9-day embryo, dorsal view. C, early 10-day embryo, dorsal view. D, 14-day pre-adult, dorsal view. E, 22-day pre-adult, dorsal view. *a*, anus; *add*, additional annulus on mouth region; *ak*, akrotroch; *am 1*, *am 2*, *am 3*, &c., amphitrochs of chaetigers 1, 2, 3, &c.; *ann*, post-prostomial annulus; *ch 1*, *ch 2*, *ch 3*, &c., chaetiger 1, 2, 3, &c.; *cha*, chaeta; *cil*, cilia; *f*, food; *gz*, growth zone; *int*, intestine; *m*, mouth; *me*, metatroch; *mes*, metatrochal segment; *mr*, mouth region; *n*, neutotroch; *oes*, oesophagus; *p*, prototroch; *pra*, proboscis apparatus; *pro*, prostomium; *py*, pygidium; *st*, stomach; *t*, telotroch.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ch 7
ch 8

FIG. 2

the body is held straight and the circlets of cilia beat in anticlockwise metachronal rhythm, diagonally backwards, so that the embryo pursues a straight course through the water, rotating on its long axis. The neurotrochal cilia, which beat directly backwards, assist in driving the embryo along.

Soon the embryo loses its negative geotropism and begins to swim at random through the water. Then, with cilia still beating, it bends itself in a half circle either to right or left, and spirals slowly to the bottom under its own weight. Once contact is made, a thigmotactic response results in ciliary gliding between the sand-grains, with some muscular bending and elongation and contraction of the body as far back as the 4th trunk segment (*ch 3*). Settling and the events which follow are not affected by the nature of the substratum. Embryos settling on sand from their natural habitat and embryos settling on clean glass showed no differences in behaviour or subsequent development.

Once the bottom-living habit is established, histo-differentiation begins. The cilia of the various ciliary bands act in unison in alternating irregular periods of metachronal activity and rest, indicating that they are under nervous control. The trunk segments become more clearly defined, the outer parts of the embryo more transparent, and muscular movements more vigorous. The embryo is, however, far from adopting the adult mode of life. The cilia of the various trochs increase in size and activity and the embryo continues to make frequent excursions through the water. Chaetae are still undeveloped, burrowing by peristalsis does not occur, and the negative phototactic response which results in burrowing in later stages of development is not yet established.

During the 8th day (figs. 1, I; 2, A) the prostomium becomes fully delimited and develops, in addition to its akrotroch, a more anterior short transverse ventral band of cilia (*cil*). The mouth region decreases in size as the prototroch becomes reduced to its two half-circles of longer cilia. All other cilia increase slightly in length and show spasmodic bursts of vigorous activity in unison, usually resulting in swimming. The main movement, however, is ciliary gliding between sand-grains. The muscular elongation and contraction which accompanies this does not assist forward progression, since the embryo, lacking chaetae, is unable to establish fixed points of contact with the substratum.

On the 9th day the first chaetae appear in rapid succession as two notopodial pairs on chaetigers 1 and 2 and a single notopodial pair on chaetiger 3 (table 1). The metatrochal segment remains without chaetae throughout development. Protrusion of the proboscis also begins on the 9th day, and this, together with the presence of the chaetae, makes it possible for the embryo to crawl over the surface of the sand-grains by muscular as well as ciliary action. Muscular crawling occurs in essentially the adult manner, by peristalsis, but is assisted by rhythmic protrusion, adhesion, and withdrawal of the proboscis. This does not occur in the adult as a locomotory activity.

Delineation of segments also recommences on the 9th day (fig. 3, A).

Within the persistent undifferentiated zone between the 4th trunk segment and the telotroch, the 5th trunk segment is demarcated, following a delay of 4 days since its components were laid down by the prepygidial growth zone.

TABLE 1

Summary of development of chaetae in H. fragilis

Day:	8	9	10	11	12	13	14	22	24	29
Segment 1	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0
,, 2	0	2	2	2	2	2	2	2	2	3
	0	0	0	1	1	2	2	2	2	3
,, 3	0	2	2	2	2	2	2	2	2	3
	0	0	0	0	1	1	2	2	2	3
,, 4	0	1	2	2	2	2	2	2	2	3
	0	0	0	0	0	0	1	1	2	2
,, 5	0	0	1	1	2	2	2	2	2	3
	0	0	0	0	0	0	0	0	1	1
,, 6	0	0	0	0	0	0	1	2	2	2
	0	0	0	0	0	0	0	0	0	1
,, 7	0	0	0	0	0	0	0	0	0	1
	0	0	0	0	0	0	0	0	0	0
,, 8	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0
,, 9	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0
,, 10	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0

The post-embryonic period

On the 10th day, although there is no decrease in ciliation, changes occur which make it possible to define subsequent development as post-embryonic and to term the developing organism a pre-adult (fig. 2, C). Feeding begins, the prepygidial growth zone (*gz*) becomes active once more, adult external features become more obvious, neurotrochal gliding ceases, peristaltic burrowing carries the embryo down into the sand, and swimming excursions become very infrequent. The last of the trunk segments laid down by the growth zone during the embryonic (lecithotrophic) period is demarcated on the 10th day (fig. 3, A) as the 6th trunk segment (chaetiger 5) after a delay of 4 days since its formation. Both it and chaetiger 4 develop amphitrochs. All remaining segments are formed after resumption of growth-zone activity and can be defined as post-embryonic. It will be seen below that the two series of segments differ not only in the time relations of their formation but also in their development. The transition from one mode of segment formation to the other is marked by a delay in growth of the last embryonic segment and also by a transient amphitroch (*am 6*, fig. 2, D), typical of the embryonic chaetigers, on the first post-embryonic segment (*ch 6*). The embryonic segments are

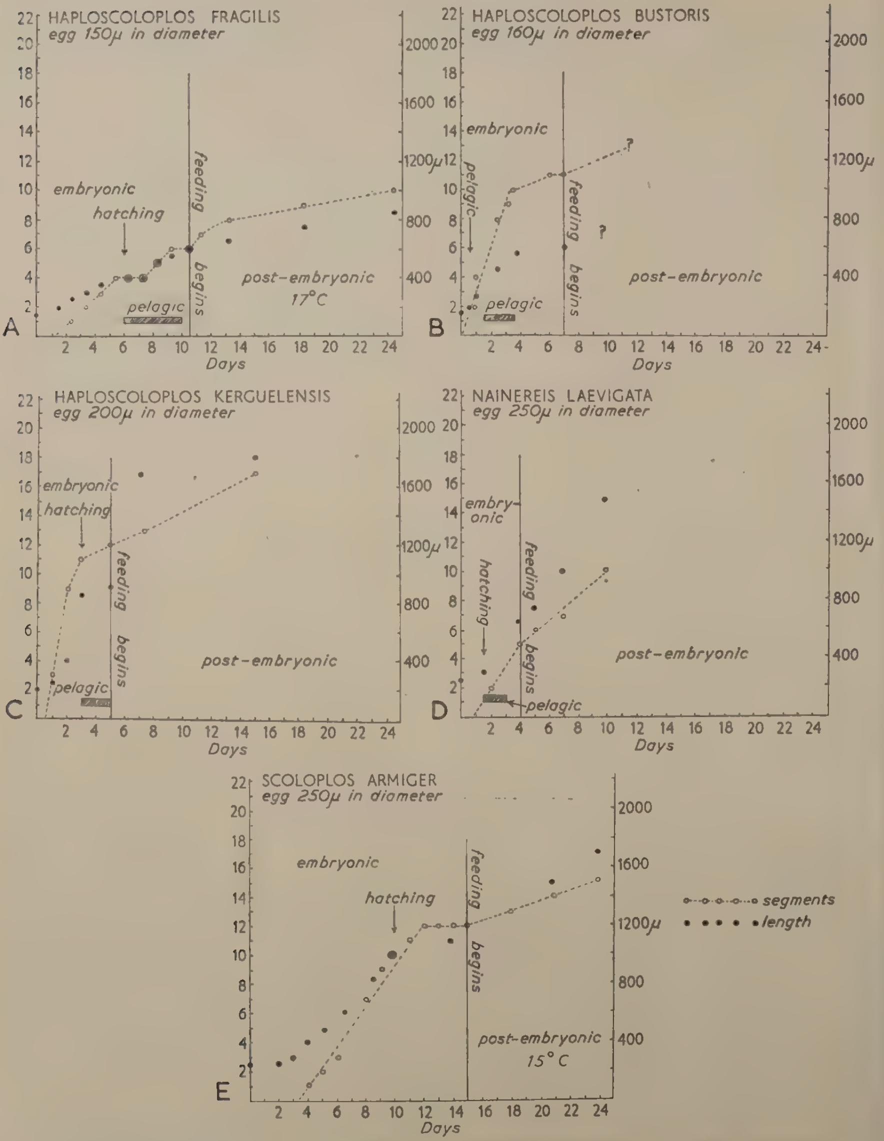

FIG. 3. External delineation of trunk segments and increase in length in developing ariciids. A, *H. fragilis*. B, *H. bustoris* (from data of Horn & Bookhout, 1950). C, *H. kerguelensis* (from data of Okuda, 1946). D, *Nainereis laevigata* (from data of Okuda, 1946). E, *S. armiger* (from data of Anderson, 1959). Ordinates: on the left, number of segments; on the right, length.

formed from material already present in the fertilized egg, the post-embryonic from material acquired through active feeding.

With the onset of post-embryonic life, overall growth slowly continues and the pre-adult (so called because its proboscis is a temporary structure replaced in the adult by a more complex proboscis) increases both in length and bulk. For the first 4 weeks of post-embryonic life the rate of increase is more or less constant. The rate at which the post-embryonic segments are formed is also constant, but differs from that obtaining during the embryonic period (fig. 3, A). The first post-embryonic segment, chaetiger 6, is delimited on the 12th day, the second on the 14th, &c. Each segment is externally delineated as soon as the material for it has been produced by the growth zone, although its subsequent development proceeds very slowly. The embryonic segments, in contrast, are slow to delineate, so that a long undemarcated zone characterizes the embryonic period; but once delineated they develop rapidly to a functional peristaltic condition. The latter is not attained by the post-embryonic segments, which thus resemble the abdominal segments of the adult and can be taken as representing the abdomen of the pre-adult. The actively peristaltic embryonic segments with their well-developed chaetae are of recognizably thoracic type. The adult, however, has 16 thoracic chaetigers. As in all ariciids, the early abdominal segments of *H. fragilis* transform to thoracic type as further segments are added to the body.

Each of the 4 components of the pre-adult body, head, thorax, abdomen, and pygidium, undergoes changes during the first 4 weeks of pre-adult life.

The head. In the adult, the head comprises the prostomium and peristomium, the latter a compound structure incorporating the mouth region and metatrochal segment of the embryo. The prostomium, once delimited, undergoes little change other than increase in size. The anterior ventral band of cilia (*cil*, fig. 2, A) is soon lost. The akrotroch persists in the pre-adult for 2 days and is then resorbed. On the mouth region, the prototroch persists until the 18th day. The neurotroch is resorbed with it (table 2) and thereafter no ciliary gliding occurs.

A more important event in the mouth region is the formation of an additional annulus (*add*, fig. 2, D), dividing the mouth region into anterior and posterior parts. The significance of this annulus, which persists through early pre-adult life, is mentioned in the discussion (p. 271).

On the metatrochal segment, the metatroch, like the prototroch, is resorbed by the 18th day. The segment then gradually fuses with the mouth region.

The thorax. By the time the first 5 chaetigers (the 2nd to 6th embryonic segments) are clearly outlined (fig. 2, C), the chaetae of the first 3 are long and a single pair of notopodial chaetae has begun to develop on the 4th. Subsequent elaboration of the thoracic segments takes place from before backwards, the first 3 showing rapid succession, the 4th and 5th being delayed in accordance with the lag in their initial development. The number of chaetae on the segments gradually increases (table 1), the notopodial being dorsolateral and the neuropodial laterally placed. All are of the simple serrate type. The amphitrochs of the thoracic segments become less conspicuous after the 12th day but are not finally resorbed until the 24th or 25th (table 2), by which time metamorphosis is complete.

TABLE 2

Summary of embryonic ciliation in H. fragilis

Days:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Antero-ventral prostomial cilia	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Akrotroch	—	—	—	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—
Prototroch	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—
Metatroch	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—
Amphitroch 1	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—
,, 2	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—
,, 3	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—
,, 4	—	—	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—
,, 5	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—
,, 6	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	—	—	—	—	—	—	—	—	—
Telotroch	—	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—
Neurotroch	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—

The abdomen. The post-embryonic segments, chaetiger 6, &c., which make up the abdomen of the pre-adult, show little change during early pre-adult life.

The pygidium. The pre-pygidial growth zone retains its simple cylindrical form in the pre-adult, but behind it the telotroch, well developed at the beginning of pre-adult life, is soon resorbed (table 2), so that the growth zone and pygidium become externally confluent. At the same time, the lateral regions of the pygidium enlarge as two lobes, leaving a median groove in which the anus, formed towards the end of the embryonic period, gradually migrates to a dorsal position.

DISCUSSION

The main interest of this study lies in the opportunity it provides for comparative discussion of the life-histories of the Ariciidae. A wide range of variation occurs within the 6 species (including *H. fragilis*) whose development is known. Comparison is made below of their eggs, modes of oviposition, pelagic phases, and ciliation. The question of the composition of the head is briefly raised as a result of the discovery of new facts pertaining to it.

The egg

The eggs of ariciids vary both in size and colour among species:

H. fragilis (this paper, p. 258)
  150 μ diameter, greenish brown-orange
H. bustoris (Horn & Bookhout, 1950)
  160 μ diameter, cream
H. kerguelensis (Okuda, 1946)
  200 μ diameter, brown
S. armiger (Anderson, 1959)
  250 μ diameter, orange
N. laevigata (Okuda, 1946)
  250 μ diameter, creamy yellow
A. foetida (Salensky, 1883)
  ? dull green-yellowish brown

In the generally accepted view, a polychaete egg greater than 160 μ in diameter undergoes non-pelagic development (Newell, 1951). This would imply a direct effect of egg size on subsequent events. The ariciids, however, do not confirm this. Although 4 of the 6 species above follow the rule, both *H. kerguelensis* and *N. laevigata*, in spite of their egg size, retain pelagic phases. The occurrence or absence of a pelagic phase in ariciid development depends on more complex factors than the dimensions of the egg.

Mode of oviposition

Among the factors associated with ariciid development, mode of oviposition appears at first sight to be unimportant. As the following table shows, mode of oviposition varies within genera and a pelagic phase may or may not follow deposition in a similar spawn:

	Oviposition	*Pelagic phase*
H. fragilis	in cylindrical gelatinous cocoons	occurs
H. bustoris	freely into water	,,
H. kerguelensis	in pear-shaped gelatinous cocoons	,,
S. armiger	in pear-shaped gelatinous cocoons	omitted
N. laevigata	in small jelly masses	occurs
A. foetida	in cylindrical gelatinous cocoons	omitted

Thorson's (1946) statement that there is no relation in polychaetes between the manner in which the eggs are spawned and the subsequent pattern of the life-history thus appears to be applicable to ariciids. Closer examination, however, reveals that this is not so. The nature of the spawn must be taken into account in explaining differences in the development of different species.

Pelagic phases in the life-history

The occurrence or omission of a pelagic phase constitutes a major difference in development among ariciids. The pelagic phase where it occurs also varies among species both in onset and duration.

	Hatches	*Becomes pelagic*	*Remains pelagic*
H. fragilis	at 6 days	at 6 days	4 days
H. bustoris	(laid freely)	on the 1st day	3½ days
H. kerguelensis	at 3 days	at 3 days	3 days
S. armiger	at 10 days	—	—
N. laevigata	at 1½ days	at 1½ days	1½ days
A. foetida	at 6 days	—	—

The only species whose eggs are laid freely, *H. bustoris*, becomes pelagic as a lecithotrophic trochophore. Other species reach a similar developmental stage on the 2nd day (compare fig. 1, B, p. 259), but are held within the jelly of the cocoon. If artificially released they are capable of ciliary gliding, and in *H. fragilis* and *H. kerguelensis* of swimming, so that the delay in escaping from the cocoon in natural conditions depends on properties of the cocoon jelly. The importance of this as a determinant of the subsequent course of development will be demonstrated below.

Ciliation

Whether or not the early stages of developing ariciids become pelagic once free in the water depends on the degree of elaboration of their ciliated bands. These may be divided into a basic series common to all species and a number of additional bands varying in arrangement among species.

The basic ciliation comprises an akrotroch, prototroch, metatroch, telotroch, and neurotroch. The prototroch always appears first, at the trochophore stage, but more important than this, the time of appearance of all bands is related to the time at which the pelagic phase of the species begins, irrespective of egg size. In order of first appearance of their basic ciliation, the 6 species under consideration arrange themselves as follows: *H. bustoris*, *N. laevigata*, *H. kerguelensis*, *H. fragilis*, *S. armiger*, *A. foetida*.

The additional ciliated bands are segmental bands lying behind the metatroch of the achaetous first trunk segment. Their development is closely linked with that of the basic ciliation, but also with the mode of formation of the trunk segments. All ariciids develop a number of embryonic segments in rapid succession; then, after a pause during which differentiation is completed, they begin to feed and to add post-embryonic segments at a slower rate (fig. 3, A–E). They show no adaptations to plankton feeding, and a pelagic phase where it occurs always lies within the embryonic period, settling being an essential preliminary to the benthic feeding of post-embryonic life. The additional ciliated bands are therefore found only on the embryonic segments.

	No. of embryonic trunk segments	*No. of segmental bands additional to metatroch*	*Settling*
H. fragilis	6 in 9 days	6 amphitrochs	10th day
H. bustoris	10 in 4 days	7 gastrotrochs	4th day
H. kerguelensis	11 in 3 days	9 amphitrochs	5th day
S. armiger	12 in 12 days	3 gastrotrochs	—
N. laevigata	3 in 3 days	none	3rd day
A. foetida	?	3 gastrotrochs	—

H. bustoris lies closest to the primitive polychaete condition. Although the pelagic phase is lecithotrophic, the freely spawned eggs become pelagic at the trochophore stage with a full basic ciliation. Owing to the availability of building material from the egg, the number of trunk segments formed during the pelagic phase is greater than the primitive polychaete number of 3 or 4 (see table above and fig. 3, B) but almost all of them develop additional segmental trochs. Presumably advantages are gained from the retention of a pelagic phase (e.g. distribution) and selection has favoured the precocious development of cilia even though other factors (e.g. a large egg) weigh against it.

In species whose eggs are spawned in jelly, there are two main evolutionary innovations—the jelly itself and in most cases a larger egg. The latter, as has already been pointed out, does not determine directly the presence or absence of a pelagic phase. *N. laevigata* has a pelagic phase, *S. armiger* does not. However, *N. laevigata* escapes from the jelly mass as an immediate post-trochophore with a fully developed basic ciliation. *S. armiger* escapes from its elaborate cocoon only after 10 days, by which time its cilia are largely resorbed. In this species the basic ciliation is not completed until the end of the 4th day. The difference between the two can be explained if it is assumed that in their common ancestors the post-trochophore stage resembled that of *H. bustoris*, but that the properties of the introduced cocoon in *S. armiger* were such as to prevent escape until the normal benthic stage had been reached. The pelagic phase being thus eliminated from the life-history, reduction in early ciliation has occurred as a corollary. The vestigial nature of additional ciliated bands of *S. armiger*, which comprise only 3 small transient gastrotrochs, confirms this. Non-pelagic development and reduced ciliation in *A. foetida* can presumably be explained in the same way. In *N. laevigata*, in contrast, in spite of an equally large egg, early escape from the simple jelly mass is associated with persistence of a pelagic phase and precociously developed cilia. The apparently anomalous absence of additional segmental trochs in this species is linked with the slow rate of delineation of the trunk segments (fig. 3, D). Growth of the trunk is not accompanied by segment delineation and the formation of additional segmental bands which would keep the embryo afloat. Settling therefore occurs early and as a corollary the embryo shows abolition of the segmental trochs.

H. kerguelensis, although it is restricted by the jelly of its cocoon for 3 days and shows some associated delay in establishing its basic ciliation, escapes at a time when pelagic activity is possible. Comparison with *H. bustoris* (fig. 3, B, C) shows that its pelagic life would be extremely brief if its cilia followed the same sequence of gain and loss as those of the latter species. Once more selection appears to have favoured maintenance of a pelagic phase, for the cilia of *H. kerguelensis* become more strongly developed than those of *H. bustoris*, 9 gastrotrochs developing before pelagic life begins. As a result, it remains pelagic for 2 days.

H. fragilis has an egg more or less equal in size to that of *H. bustoris* and a trochophore which can swim if artificially released; yet the embryo of this species does not hatch for 6 days. The delay can be ascribed partly to a slow rate of development (compare fig. 3, A, B) but also to the fact that the cocoon is more retentive than those of *N. laevigata* and *H. kerguelensis*. The basic ciliated bands are slow to develop, as in *S. armiger* and *A. foetida*, presumably due to the selective action of the cocoon environment; but escape still occurs at a time when the embryo has, relatively to its size, a ciliation sufficient for pelagic activity. As in *H. kerguelensis*, selection in the post-hatching stages of development appears to have been towards retention and elaboration of the cilia, prolonging pelagic life for a further 4 days. The 5 amphitrochs which develop on the embryonic segments confirm this. When settling occurs at 10 days, however, the cilia are still fully developed, being finally resorbed only on the 24th day. The explanation of early settling is to be found in the food requirement of the developing organism, which has utilized its yolk reserves by 10 days and has no special apparatus for plankton feeding; but this does not account for the extraordinary persistence of the ciliated bands after settling. Why they should be retained in *H. fragilis* when in other species they are rapidly lost once benthic life begins remains a mystery.

In general it can be concluded that in species whose embryo becomes free in the water at a stage sufficiently ciliated to be pelagic, natural selection has favoured retention and elaboration of cilia and a pelagic phase is maintained irrespective of egg size. Where the properties of the gelatinous cocoon have been such as to preclude escape before the onset of metamorphosis, the resulting elimination of the pelagic phase has led to reduction of the ciliated bands. The occurrence or absence of a pelagic phase in ariciid development thus depends in part on the properties of the cocoon, i.e. on mode of oviposition, the most important property being probably the viscosity of the jelly. A detailed study of changes in jelly viscosity in the cocoons of different species between oviposition and hatching would be of particular interest.

Development of the head

In every species of ariciid so far examined, the delineation of the first trunk segment also results in demarcation of an anterior prototrochal region. The posterior half of this bears the prototroch and the mouth. The anterior half enlarges, a pair of dorso-lateral eye-spots appears in front of the anterior margin of the prototroch, and an annulus formed just behind the eyes then delimits an anterior akrotroch-bearing prostomium from a posterior prototroch-bearing mouth region.

The prostomium, which was shown by Anderson (1959) to be a pre-segmental body unit, undergoes little further change other than increase in size and loss of the akrotroch. In *N. laevigata* and *H. kerguelensis* the eyes regress during early pre-adult life but in other species they persist into the adult.

The mouth region, conversely, decreases in size as the prototroch regresses, while ventrally in front of and behind the mouth superficial folds develop as the upper and lower lips. Anderson (1959) adduced reasons for regarding the mouth region, as well as the prostomium, as a pre-segmental structure. In connexion with this, further events which take place in the mouth region of *H. fragilis* and also in *H. kerguelensis* are of interest. In *S. armiger* (Anderson, 1959) it was shown that 3 pairs of mesodermal somites initially invade the mouth region while it is still part of the prototrochal region, but that they do not induce a corresponding segmentation in the ectoderm of this region. Furthermore, no ventral nerve ganglia are associated with them, the first ganglion of the ventral nerve-cord lying in the metatrochal segment and subsequently migrating back to fuse with that of chaetiger 1. For these and other reasons, it was decided that the mouth-region somites of *S. armiger* could not be regarded as the somites of cephalized segments and that the polychaete head bore no obvious correspondence to the onychophoran-arthropod head. In *H. fragilis* (p. 265) and *H. kerguelensis*, however, an additional annulus arises on the mouth region after it has been delimited from both the prostomium and the metatrochal (first trunk) segment, dividing the mouth region into anterior (mouth-bearing) and posterior parts. In external appearance the latter resembles an achaetous segment. The question therefore arises, does it correspond to any of one or more pairs of somites which invade the mouth region in either of these species, and does it show any signs of developing an ectodermal segmental ganglion as the first ganglion of the ventral nerve-cord? If so, i.e. if it can be recognized as a cephalized segment, Anderson's (1959) view that the mouth region in ariciids and other polychaetes is definably presegmental will have to be revised, since it becomes possible that cephalization may have embryological consequences which lead to the segments concerned being recognizable only by the existence of a pair of mesodermal somites. Such a change of viewpoint would have considerable importance in the interpretation of segmental composition in metamerically segmented animals. It is hoped shortly to carry out the detailed embryological study which will confirm or deny this hypothesis.

Whether or not the mouth region becomes subdivided, the subsequent history of the adult peristomium is similar in all ariciids studied. The peristomium arises by fusion of the achaetous metatrochal segment with the mouth region. There are thus no grounds for denying that the peristomium in ariciids is a complex structure including at least one cephalized trunk segment in its composition.

I wish to express my thanks to Miss I. Bennett for advice on the collection of material, to Prof. P. D. F. Murray and Dr. L. C. Birch for valuable discussion, to Dr. L. E. R. Picken for his critical appraisal of the manuscript, and to the University Grants Committee of the University of Sydney for financial aid.

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The ‘Chromatoid Body’ in Spermatogenesis

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SUMMARY

The chromatoid body was discovered by von Brunn (1876) in the cytoplasm of the young spermatid in the white rat. It was first described in a marsupial by Korff (1902), in a vertebrate other than mammals by the Schreiners (1905, 1908), and in an invertebrate by Bösenberg (1905).

The word *chromatoide* was first used in connexion with spermatogenesis by Benda (1891), who called this cytoplasmic inclusion *der chromatoide Nebenkörper*. The German authors generally call it *der chromatoide Körper*, the French authors *corps chromatoïde*. Wilson (1913) referred to it as the chromatoid body and it is generally given this name in papers written in English, though the expression ‘chromatic body’ is sometimes used. It is suggested that the ‘residual body’ described by Gresson and Zlotnik (1945) is identical with the chromatoid body of other authors.

In most species the chromatoid body is spherical or ovoid but in some it assumes other forms as well and in a few it is never spherical or ovoid.

The chromatoid body is usually single in each cell, but sometimes there are 2 or 3 and in a few there are many.

In living cell the chromatoid body generally gives a low phase-change, and is invisible or almost invisible when studied by direct microscopy. In the Mammalia, however, it gives a higher phase-change.

The chromatoid body is highly resistant to acetic acid.

It is deeply stained by basic dyes and basic dye-lakes. It is also stained intensely by acid dyes.

The chromatoid body cannot in most cases be blackened by silver or long osmication techniques.

The histochemical reactions show that the chromatoid body consists mainly of

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RNA and basic proteins rich in arginine. There is little or no tyrosine. Lipid, carbohydrates, DNA, alkaline phosphatase, and calcium are not shown by histochemical techniques.

As a rule the chromatoid body is homogeneous but in some cases it has a cortex and a medulla. In many cases it is surrounded by a clear, vacuole-like space. Under the electron microscope it has been seen as an opaque irregular body, as an irregular mass of closely aggregated, dense, osmiophil granules, or as a faintly electron-opaque body.

The chromatoid body has so far been recorded in certain species of mammals, a bird, reptiles, cyclostomes, Crustacea, insects, and arachnids. In most cases it appears for the first time during the growth of the primary spermatocyte. Its presence in the spermatid has been recorded in practically all cases. With a few exceptions it has not been found to take any obvious part in the final make-up of the spermatozoon.

The chromatoid body in most cases seems to disappear at the metaphases of meiosis and to be later reconstructed in the daughter cells.

The chromatoid body probably originates from the ground cytoplasm.

On the basis of histochemical studies it is tentatively suggested that the function of the chromatoid body may be to provide basic proteins for the final maturation of the chromatin in the nucleus of late spermatids.

Certain authors have considered that a cytoplasmic inclusion occurring in the young (and in some cases mature) spermatozooids of certain liverworts, mosses, and a gymnosperm is to be regarded as the homologue of the chromatoid body. Reasons are given for denying this supposed homology.

Introduction

PROMINENT cytoplasmic inclusions of unknown significance have been described under the name of chromatoid bodies in the male germ-cells of certain mammals, a bird, reptiles, cyclostomes, Crustacea, insects, and arachnids. In its characteristic form the chromatoid body is a large, spherical or subspherical inclusion in spermatocytes and spermatids. It often appears faint in living cells, even when examined by phase-contrast microscopy, but it stains intensely with basic as well as acid dyes and is highly resistant to acetic acid. Generally there is only one in each cell. It does not appear to make any visible contribution to the final make-up of the spermatozoon.

Discovery

The chromatoid body was discovered in the young spermatid of the white rat. In this cell it was figured in 1876 by von Brunn (fig. 1), who did not mention it, however, in the text of his paper. Hermann (1889) described in the primary spermatocyte of the mouse a small *Knöpfchen*, stainable with gentian violet; he distinguished it from the colourless idiozome. This may have been a chromatoid body. Benda (1891) studied testicular material of the rat, mouse, cavy, hedgehog, and other mammals, and distinguished the chromatoid body in their spermatocytes. Niessing (1897) saw it in the primary spermatocytes and spermatids of the rat (fig. 2, A, B) and guinea-pig (C, D), and in spermatids of the mouse. Schœnfeld (1900) recorded its presence in the primary and secondary spermatocytes of the bull (E). Korff (1902) described it in the spermatid of the marsupial, *Phalangista vulpina*.

The Schreiners (1905, 1908) were the first to demonstrate the chromatoid

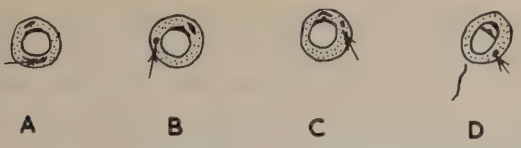

FIG. 1. A–D, developing young spermatids of the white rat (copied from Brunn, 1876). Arrows have been inserted by the author of the present paper to indicate the chromatoid body.

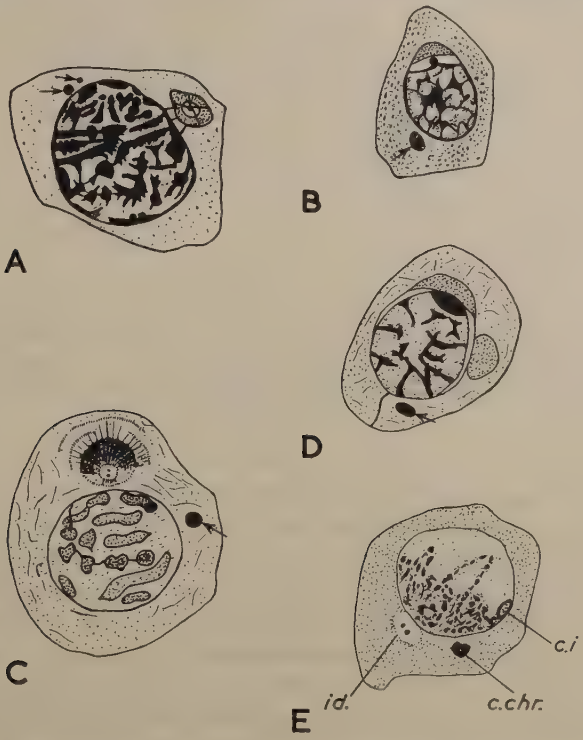

FIG. 2. A, primary spermatocyte (*Mutterzelle*) and B, spermatid of the rat. C, primary spermatocyte (*Mutterzelle*) and D, spermatid of the guinea-pig (copied from Niessing, 1897). E, primary spermatocyte of the bull (copied from Schœnfeld, 1900). *c.chr.*, chromatoid body (arrows have been inserted to indicate the chromatoid body in those figures in which the original author did not label it). *c.i.*, *corpuscules intranucléaire. id.*, idiozome.

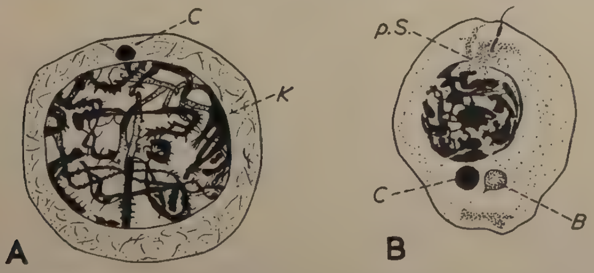

FIG. 3. A, primary spermatocyte (copied from the Schreiners, 1905) and B, young spermatid (copied from the Schreiners, 1908) of *Myxine glutinosa. B, Sphärenbläschen. C*, chromatoid body. *K, Knotenkörper. p.S., primäres Spitzenbläschen.*

body in vertebrates other than mammals. They saw it in the primary and secondary spermatocytes and spermatids of *Myxine glutinosa* (fig. 3, A, B), and in the primary and secondary spermatocytes of *Bdellostoma burgeri*. It was first seen in invertebrates by Bösenberg (1905), who recorded it in the spermatid of the spider, *Lycosa* sp. (fig. 4, A), and by Schäfer (1907) in the primary spermatocytes and spermatids of the beetle, *Dytiscus* sp. (fig. 4, B).

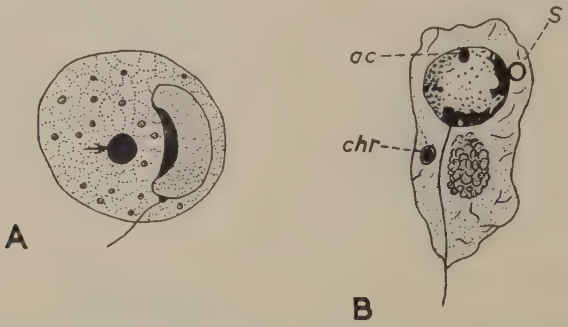

FIG. 4. A, spermatid of *Lycosa* sp. (copied from Bösenberg, 1905). B, spermatid of *Dytiscus* sp. (copied from Schäfer, 1907). *ac, akzessorisches Chromosom. chr*, chromatoid body (an arrow has been inserted to indicate the chromatoid body where it was not labelled by the original author). *S, Sphäre.*

NOMENCLATURE

The word *chromatoide* was first used in connexion with spermatogenesis by Benda (1891) in his study of the testis of various mammals. He described *der chromatoide Nebenkörper* in the spermatocytes (*Mutterzellen*). He took the word *Nebenkörper* from Hermann (1889), who in fact applied it to a complex in which he included the idiozome and centrioles. Benda distinguished under the name *der chromatoide Nebenkörper* a body in the spermatocytes of mammals that stains with safranine. This is a character that distinguishes it from the idiozome and centrioles. The chromatoid body of modern cytology has no connexion with centrioles.

Niessing (1897), Lenhossék (1898), Meves (1899), Korff (1902), and Bösenberg (1905) used Benda's terminology. The Schreiners (1905, 1908) and Schäfer (1907) used the expression *der chromatoide Körper*. The Schreiners, however, used the term *die chromatoiden Körperchen* for the smaller bodies which fuse to form *der chromatoide Körper*.

Schœnfeld (1900), van Molle (1906), Duesberg (1908, 1909, 1911), Regaud (1910), and von Winiwarter (1912) called it *corps chromatoïde*.

Earlier Moore (1893, 1894) called it 'chromatic body'. Austin and Sapsford (1951) and Burgos and Fawcett (1955) have used the same name. Wilson in 1913 called it chromatoid body and it is usually referred to by this name in papers written in the English language.

Fox (1952) figured the chromatoid body in the late spermatids of the garter snake, *Thamnophis*, but wrongly named it 'acroblast'.

Gresson and Zlotnik (1945, 1948) in eight species of the mammals from the orders Lagomorpha, Rodentia, Carnivora, and Artiodactyla, and Zlotnik (1947) in the domestic fowl, *Gallus domesticus*, have used the name 'accessory bodies' for cytoplasmic inclusions in the male germ-cells which they consider to be the same as what other authors have called chromatoid bodies. These accessory bodies originate from the localized Golgi material and are argentophil (Gresson and Zlotnik, 1945; Zlotnik, 1947) or osmiophil (Gresson and Zlotnik, 1948). The term 'accessory body' was first used by Gatenby and Beams (1935) for a structure present in the mammalian spermatocyte. Gatenby and Beams are inclined to the view that many of the 'accessory bodies' are parts of the spermatogonial Golgi elements which fail to collect together, but they feel that there is still a possibility that they are something different. Sharma and others (1956) also find that the 'accessory body' in *G. domesticus* comes out of the localized Golgi material and, therefore, they conclude that it seems to be a part of the latter. Sud (1955) and Sharma and Gupta (1956) are of the opinion that the chromatoid body is chemically different from the Golgi elements. Tobias (1956) has reason to believe that Gresson and Zlotnik (1945) include at least two different categories of inclusions in their class of accessory body, and he is of the firm opinion that the chromatoid body is an element distinct from the accessory body of Gatenby and Beams. I am inclined to the view that the 'residual body' described by Gresson and Zlotnik (1945) in the white rat, *Rattus norvegicus albinus*, and the Golden hamster, *Cricetus auratus*, is identical with the chromatoid body described by Moore (1893, 1894), Niessing (1897), Lenhossék (1898), Duesberg (1909), and Regaud (1910) in the spermatid of the rat. The 'residual body' of Gresson and Zlotnik stains with iron haematoxylin and acid fuchsine like the 'accessory body', but differs from the latter in not being argentophil or somiophil. Further, whereas an 'accessory body' is present in the neck region of the spermatozoon, the 'residual body' breaks up in the residual cytoplasm. For the reasons cited above, the 'accessory bodies' of Gatenby and Beams (1935), Gresson and Zlotnik (1945, 1948), Zlotnik (1947), and Gresson (1948, 1950) will not be mentioned in the subsequent pages of this review.

SHAPE

The chromatoid body has mostly been described as a spherical or ovoid body. In the guinea-pig, Meves (1899) and Fawcett and Ito (1958) describe it as an irregularly shaped body, and Gatenby and Woodger (1921) figure it in various shapes other than spherical. In the rat, according to Niessing (1897), the chromatoid body during late stages of spermateleosis undergoes changes in form: it may be rounded or elongated or angular. Lenhossék states that in the spermatid of the rat the chromatoid body is spherical but sometimes elongated into an ellipsoid, or it may be like two spheres fused together. Duesberg (1908) in the albino rat found in the secondary spermatocyte a chromatoid body of irregular form, often bilobed or doubled, and in the spermatid (1909) he saw it as an irregular, lobulated body; but during the late stages of spermateleosis he found that it regularly becomes spherical. In the squirrel, van Molle (1906) found it elongated and twisted in the spermatid but spherical in the secondary spermatocyte. In *Myxine* the Schreiners (1908) found that the chromatoid body is usually of irregular form. It is often dumbbell-shaped or consists of two or three or more spheres, of which one is markedly larger than the others and connected with them by fine bridges. In the latter condition the chromatoid body appears to be provided with many projections turned in the same direction, usually towards the cell periphery or the nucleus.

In the snake, *Natrix piscator*, I (Sud, 1955) found that the chromatoid body when it reappears during spermateleosis is at first dumbbell-shaped but later becomes spherical. In man, von Winiwarter (1912) found *corpuscules chromatophiles* which might aggregate into a *bâtonnet*. Pollister (1930) in *Gerris* (Hemiptera-Heteroptera) states that the chromatoid bodies are usually curved rods, somewhat sickle-shaped.

NUMBER IN EACH CELL

In most animals there is a single chromatoid body in each cell, but in some cases more than one have been recorded. In the rat, Niessing (1897) figured two chromatoid bodies in the primary spermatocyte, and Lenhossék (1898) and Regaud (1910) recorded one to three, generally two, chromatoid bodies during the growth of the primary spermatocyte. In the albino rat, Allen (1918) recorded a double chromatoid body in some metaphases of the primary spermatocyte; Koller and Darlington (1934) also saw two small chromatoid bodies in the first meiotic division. Duesberg (1908) states that all authors agree that the chromatoid body of the rat may fragment. In the gerbil, *Tatra brantsii*, Tobias (1956) found one or more chromatoid bodies. In man von Winiwarter (1912) recorded a certain number of *corpuscules chromatophiles* which might coalesce to form a *bâtonnet*. Small additional chromatoid bodies have been recorded in some cases: one or two in the horse (Wodsedalek, 1914), one or two or more in the snake (*N. piscator*) and turtle (*Lissemys punctata*) (Sud, 1955, 1956). In the crayfish *Cambarus virilis* (Fasten, 1914, McCroan, 1940), the crab *Cancer magister* (Fasten, 1918), *Gerris* (Hemiptera-Heteroptera) (Pollister, 1930), and the lizard *Uromastix* (Sud, 1957), two large chromatoid bodies are seen in some stages of spermatogenesis. In the young spermatid of the spider *Lycosa* (Bösenberg, 1905) there are one or two chromatoid bodies. In the beetle *Dytiscus*, Schäfer (1907) figures three chromatoid bodies in one of the spermatids. In *Lepisma domestica* several deeply staining granules have been considered by Bowen (1924) as possibly related to the chromatoid body, but Gatenby and Mukerji (1929) describe a single large, spherical chromatoid body in the spermatid of this species.

APPEARANCE IN LIVING CELL

In living material Plough (1917) found that the chromatoid body was practically invisible in unstained cells. Pollister (1930) also states that the chromatoid bodies are not visible in fresh preparations. According to Austin and Sapsford (1951) the 'chromatic body' in the living rat spermatid, under phase contrast, is darker and generally more compact than the Golgi apparatus and is finely granular. Fawcett and Ito (1958) describe the chromatoid body as a dense mass in living spermatid of the guinea-pig under phase contrast. Gupta (1955), Sharma and Gupta (1956), and I (Sud, 1955, 1956, 1957, 1961 *a*, *b*) found that the chromatoid body in living cells gives a very low phase-change. I further found that in the snake, *N. natrix*, the chromatoid body in the spermatid gives a slightly higher phase-change than in the spermatocyte though it is much smaller, and thus concluded that there is a higher proportion of solid material in the chromatoid body of the spermatid (Sud, 1961 *b*).

Sharma and Gupta (1956) and I (Sud, 1961 *a*, *b*) are of the opinion that the chromatoid body does not take up neutral red, but Plough (1917) found that it appears as a highly refractile pink drop after a rather long treatment with neutral red. It is not stained by Janus green (Sud, 1961 *a*, *b*).

STAINING REACTIONS IN FIXED PREPARATIONS

The chromatoid body has mostly been studied in material fixed in mixtures containing acetic acid. The chromatoid body is highly resistant to Bouin's, Carnoy's, and Zenker's fluids and is not even slightly corroded in such preparations (Sud, 1955, 1956, 1957, 1958, 1961*b*). Dhillon (unpublished, quoted by Nath, 1957) also found that the chromatoid bodies are not washed out by Carnoy's fluid. Gupta (1955) states that the chromatoid body is highly resistant to acetic acid.

It is characteristic of the chromatoid body that it stains with basic dyes such as safranine (Benda, 1891; Regaud, 1910; Wilson, 1913; Plough, 1917; McCroan, 1940; Sud, 1961*b*) and crystal violet (McCroan, 1940; Tobias, 1956; Sud, 1961*b*). I (Sud, 1961*b*) have also been able to stain it intensely by aqueous basic fuchsine, pyronine in the pyronine / methyl green technique (Jordan and Baker, 1955), and cresyl violet in the Nissl technique (Fernstrom, 1958). Moore (1894) found the chromatoid body to stain bright red in sections first treated with a mixture of (basic?) fuchsine and methyl green and later with orange G to wash out the double stain thus formed. According to Lenhossék (1898) it stains with gentian in the gentian / safranine mixture or with magenta (basic fuchsine). He further states that certain nuclear dyes, such as thionine and methylene blue, do not stain it in spermatocytes. The Schreiners (1905) in *Myxine* found that the chromatoid bodies (*die chromatoiden Körper*) are formed from chromatoid corpuscles (*chromatoiden Körperchen*) which come out of the nucleus at the beginning of the period of maturation of the primary spermatocyte. The chromatoid corpuscles, while in the nucleus or on their way out and while fusing, stain with methylene blue, but the large chromatoid bodies formed from them stain bright red with eosin in methylene blue / eosin preparations. The Schreiners (1908) believe that this change from basiphilia to esinophilia begins already in the nucleus and ends in the cytoplasm. When the chromatoid body moves back into the nucleus in the spermatid stage no change in the staining reaction occurs. Regaud (1910) states that the chromatoid body is coloured by all basic aniline dyes.

In general, the chromatoid body stains deeply with basic dye-lakes. Most workers on the chromatoid body, using different methods of fixation and mordanting, have recorded that it takes up iron haematoxylin deeply (Lenhossék, 1898; the Schreiners, 1905; Regaud, 1910; Wilson, 1913; Plough, 1917; Pollister, 1930; McCroan, 1940; Bishop, 1942; Sud, 1955, 1956, 1957, 1961*b*; Gupta, 1955; Sharma and Gupta, 1956; Tobias, 1956). But Grabowska (1929) describes it as an unstained sphere in Hirschler preparations. Regaud (1910) states that haemalum colours the chromatoid bodies in pale violet like the nucleoli. According to Plough (1917) the chromatoid body and the nuclei both appear bright purple with Benda's alizarin / crystal violet method. Duesberg (1911) found that the chromatoid body in the guinea-pig is coloured brown just like the nucleolus by Benda's method. Pollister (1930) also states that the chromatoid body stains with alizarin by this method.

The chromatoid body in many cases stains deeply with certain acid dyes, especially acid fuchsine (Meves, 1899; Plough, 1917; Ludford and Gatenby, 1921; Pollister, 1930; Sud, 1955, 1956, 1958 1961*b*; Gupta, 1955) and azocarmine (Sud, 1961*b*). Plough (1917) found that with Auerbach's rubin (acid fuchsine) / methyl green mixture the chromatoid body stains bright red while the nuclei are green. Grabowska (1929), on the contrary, describes the chromatoid body as unstained sphere in Altmann mitochondrial preparations.

DARKENING BY SILVER AND OSMIUM

There are very few accounts of the darkening of the chromatoid body by metals. In Kolatchev preparations of the testis of *Cavia* Gatenby and Wigoder depict the chromatoid body of normal and giant spermatids as a duplex spherical or subspherical structure with a thick dark rim and a clear central region. McCroan (1940) found that the chromatoid bodies in the crayfish, *Cambarus virilis*, occasionally blacken lightly with osmium and silver. Contrary to these observations, in the reptiles (Sud, 1955, 1956, 1957, 1958, 1961*b*) and domestic duck (Gupta, 1955) the chromatoid body does not blacken by silvering or long osmication techniques.

CHEMICAL COMPOSITION

Histochemical tests do not show lipid, carbohydrates, alkaline phosphatase, calcium (Sud, 1961 *a*, *b*) or DNA (Schrader, 1940; Gupta, 1955; Sud, 1961 *a*, *b*) in the chromatoid body. Its intense basiphilia is due to RNA (Daoust and Clermont, 1955; Sud, 1961 *a*, *b*), but probably also in part to acidic groups in protein (Sud, 1961 *a*, *b*). It gives a positive reaction with the mercuric / bromophenol blue technique (Sud, 1958). The intense acidophilia of the chromatoid body indicates the presence of basic proteins (Sud, 1961 *a*, *b*). It gives an intensely positive Sakaguchi reaction of Baker (1947) which presumably shows the presence of protein-bound arginine (Sud, 1961 *b*).

After deamination the chromatoid body is negative to the Sakaguchi reaction but there is no change in its intense acidophilia. It appears that the acid dyes must be reacting with basic groups other than $-NH_2$ (Sud, 1961 *a*, *b*).

The chromatoid body gives a negative result with the alkaline fast green reagent of Alfert and Geschwind (1953); this suggests the absence of histones. The negative result with alkaline fast green suggests that the arginine occurs mostly as a component of protamines rather than histones (Sud, 1961 *a*, *b*). The chromatoid body contains little or no tyrosine (Sud, 1961 *a*, *b*).

INTERNAL STRUCTURE

As a rule chromatoid bodies are homogeneous. Niessing (1897), while discussing the chromatoid bodies in the rat and mouse, states that he has often been able to distinguish in this body a lighter and a darker component. The figures of Gatenby and Woodger (1921) and Gatenby and Wigoder (1929) suggest that the chromatoid bodies in *Cavia* consist of a cortex and a medulla. Koller and Darlington (1934) figure both homogeneous and duplex chromatoid bodies in the albino rat. Koller (1936*b*) in the ferret, *Putorius furio*, and mole, *Talpa europea*, states that the chromatoid body appears to be composed of two differently stained parts. Tobias (1956) in the gerbil, *Tatera brantsii*, found that the chromatoid body usually possesses a non-staining or lightly staining core. Wilson (1913) in *Pentatoma* (*Rhytodolomia*) *senilis* (Hemiptera-Heteroptera) frequently found in the chromatoid body more or less definite indications of a central cavity, with occasionally a sharply stained central granule inside it. Moore (1893, 1894) in the rat describes it as a granular mass. Austin and Sapsford (1951) in living spermatids of the rat found the 'chromatic body' finely granular in texture and generally more compact than the Golgi apparatus (p. 279).

A number of workers have described a clear, vacuole-like space surrounding the chromatoid body (Bösenberg, 1905; Schäfer, 1907; the Schreiners, 1908; Wilson, 1913; Wodsedalek, 1914; Fasten, 1914, 1918; Plough, 1917; McCroan, 1940; Gupta, 1955).

Under the electron microscope, Watson (1952) observed the chromatoid body in the rat spermatid as an opaque, irregular body, and Burgos and Fawcett (1955) have described the 'chromatic body' in the cat spermatid as an irregular mass of closely aggregated, dense, osmiophil granules. In *Nemobius* (Gryllidae) Gatenby and Tahmisian (1959) found the chromatoid body faintly electron-opaque. Swift (1956), in electron micrographs of the rat spermatid, found small lamellar arrays associated with the chromatoid body.

OCCURRENCE

The presence of the chromatoid body in the spermatogenesis of different species, as worked out by various authors, is shown in tabular form in the appendix (p. 290). It will be found convenient to have this bird's-eye view of the presence or absence, and time of appearance and disappearance, of the chromatoid body in different species at various stages of spermatogenesis.

This table shows that the chromatoid body has mostly been studied in mammals, particularly rodents. Some data are also available from other eutherian mammals, Insectivora, Primates, Carnivora, Perissodactyla, and Artiodactyla. Only fragmentary information is available about Metatheria. Among other vertebrates the chromatoid body has been studied in the domestic duck among Aves, in *Lissemys*, *Uromastix*, *Natrix*, and *Thamnophis*, representing the major groups of Reptilia, and in *Myxine* and *Bdellostoma* among Cyclostomata. In the invertebrates it has been studied mostly in insects of the orders Thysanura, Orthoptera, Hemiptera-Heteroptera, and Coleoptera. It has also been found in *Pardosa* and *Lycosa* among Arachnida, and in *Cambarus*, *Potamobius*, and *Cancer* among Crustacea.

From the table it appears that in most cases the chromatoid body appears for the first time during the growth of the primary spermatocyte. Its presence in the spermatid stage has been recorded in practically all cases, and with a few exceptions it has not been found to take part in the final make-up of the spermatozoon.

BEHAVIOUR DURING DIVISION STAGES

Varied accounts have been given of the behaviour of the chromatoid body during the division stages. Wilson (1913), Wodsedalek (1914), Plough (1917), Koller (1936*c*), Long (1940), Schrader (1940), and Bishop (1942) find that the chromatoid body is carried through both the meiotic divisions but does not divide and thus enters only one of the four spermatids. Fasten (1914, 1918) also believes that the chromatoid bodies do not divide and pass as such to the daughter cells.

According to Lenhossék (1898), in the rat the chromatoid bodies are present during the first meiotic division and are carried over into the secondary spermatocytes with increase in size. One chromatoid body is present in each secondary spermatocyte and each spermatid. He thinks that the chromatoid body in the spermatid is perhaps derived from that of the primary spermatocyte but is larger in the spermatid. Niessing (1897) also is of the opinion that the chromatoid bodies in the spermatids of the guinea-pig are derived from that of the primary spermatocyte.

Division of the chromatoid body into two equal parts and the distribution of the latter to the daughter cells at metaphase has been observed during both the meiotic divisions in *Rattus* ('*Mus*') *rattus* and *Cavia* by Ludford and Gatenby (1921). Earlier, Duesberg in the albino rat (1908) and guinea-pig (1911) found that the chromatoid body appears first in the secondary spermatocyte and divides during the second meiotic division. Koller and Darlington (1934) in the albino rat regularly found the chromatoid body at the metaphase and anaphase of the second meiotic division and sometimes saw it as though dividing. In the first meiotic division they observed two much smaller bodies and believe that the bodies seen at second division might be derived from these. Gupta (1955) found that the chromatoid body in the domestic duck divides into two during cell-divisions. Tobias (1956) in the gerbil, *Tatera brantsii*, has shown that the chromatoid body is capable of growth, fusion, and division, and that there is a definite genetic continuity of the chromatoid body of the primary spermatocyte and spermatid.

Disappearance of the chromatoid body at the time of cell-division and its subsequent reconstruction has been reported by Schœnfeld (1900) in the bull and by Regaud (1910) in the rat. Schœnfeld records that it disappears at metaphase I and reappears in the young secondary spermatocyte, from which it is eliminated. According to Regaud the chromatoid bodies disappear during the first meiotic division and reconstruct themselves into a single body in each secondary spermatocyte. During the second meiotic division the chromatoid body reduplicates into granules which divide themselves between the daughter cells.

The Schreiners (1905, 1908) found in *Myxine* that during the meiotic divisions the chromatoid body appears looser in texture and less sharply defined and in telophase becomes sharply defined again; occasionally it is represented by large and small granules. The chromatoid bodies are divided between the daughter cells. In each succeeding generation of cells, from primary spermatocyte to spermatid, the size of the chromatoid element is reduced to half.

I did not find chromatoid bodies during meiotic metaphases in the turtle *Lissemys punctata* (Sud, 1956), or in the snake *Natrix natrix* (Sud, 1961 *a*, *b*), though I recorded their presence in the resting primary and secondary spermatocytes and late spermatids.

ORIGIN

Conflicting views have been expressed as to the origin of the chromatoid body. Benda (1891) and Regaud (1910) are inclined to the view that the chromatoid body is derived from the nucleus. Moore (1893, 1894) and the Schreiners (1905) say definitely that the chromatoid body in their material is formed by the aggregation of particles derived from the nucleus. But Lenhossék (1898) states that the chromatoid body does not consist of ordinary chromatin since characteristically nuclear dyes, such as thionine and methylene blue, do not stain it in spermatocytes. Fasten (1918) also found that nuclear dyes are more easily extracted from the chromatoid bodies than from the chromatin of the nucleus.

It is claimed by Ebner (1899), Fischer (1899), and Duesberg (1908, 1911) that the chromatoid body is derived from the nucleolus. Lenhossék (1898) and Schäfer (1907) also consider this quite likely. Von Winiwarter (1912), on the contrary, states that the hypothesis of the nucleolar origin of the chromatoid body is rendered unlikely by the fact that in man the spermatogonia have no nucleolus but are provided with chromatoid corpuscles.

Plough (1917) believes that the chromatoid body in the grasshopper, *Rhomaleum*, is formed from a specific substance which is a normal inclusion of the cytoplasm of certain somatic and 'apical cells'. This substance is present in small quantities in the cytoplasm of the spermatogonium and young primary spermatocyte of some grasshoppers but increases in amount during the growth period and may collect into one mass, which like any other foreign substance in the cytoplasm appears to lie in a vacuole in fixed material.

Wilson (1913) claims that in *Pentatoma* (Hemiptera-Heteroptera), the chromatoid body originates from the ground cytoplasm and is not derived from centrosome, centriole, acrosome, or extruded nucleolus. In the reptiles, *Natrix*, *Lissemys*, and *Uromastix*, from the reaction of the chromatoid body to various fixing and staining media coupled with its study in the living material under phase contrast, I (Sud, 1955, 1956, 1957) concluded that the chromatoid body appears to be an independent cell structure having a direct origin from the ground cytoplasm. Pollister (1930) remarks that the chromatoid bodies in Gerris (Hemiptera-Heteroptera) are not related to any other cytoplasmic structure. Tobias (1956) also concludes that the chromatoid bodies in the gerbil, *Tatera brantsii*, are not formed from any other specific cell inclusion.

DISAPPEARANCE

The chromatoid body can usually not be seen in the ripe spermatozoon. In the rat (Austin and Sapsford, 1951), horse (Wodsedalek, 1914), fox (*Vulpes fulva*) (Bishop, 1942), domestic duck (Gupta, 1955), and *Pentatoma* (Hemiptera-Heteroptera) (Wilson, 1913), the chromatoid body is sloughed off with the residual cytoplasm during spermateleosis. The chromatoid body in the spermatid of the crab, *Cancer magister* (Fasten, 1918), when present, moves to the periphery of the cell and is soon expelled to the outside. In the marsupial, *Phalangista vulpina* (Korff, 1902), and the beetle, *Dytiscus* (Schäfer, 1907), the chromatoid body first resolves itself into granules during spermateleosis and these are sloughed off with the residual cytoplasm.

In the albino rat (Duesberg, 1909) the chromatoid body becomes gradually smaller during the final stages of spermateleosis and then fragments into two or three pieces, each of which dissolves in turn insensibly in the cytoplasm. But from Regaud's (1901 *a*, *b*) description of spermatogenesis of the rat it is evident that in the late stages of spermateleosis the chromatoid body fragments into several spherules of unequal size which he calls *la substance chromatoide* and are homologous with von Ebner's (1888) *die tingirbaren Körnchen*. These again come together to form a single *sphère chromatophile* in the discarded residual body and later assume the aspect of *une châtaigne épineuse* (Regaud, 1901*b*). Smith and Lacy (1959) also describe decrease in number and increase in size of the basiphil granules during the late stages of spermateleosis of the rat and they further state that the greater part of each residual body is occupied by a large, eccentrically placed spheroidal mass of strongly basiphil material consisting of RNA and appearing as particulate masses in electron micrographs. They, in conformity with Regaud (1901*b*), found that the residual bodies are phagocytosed by the Sertoli cells. On reaching the edge of the tubule the limiting membranes of the residual body seen by electron microscopy are no more continuous and its basiphil material and various other components are absorbed, leaving behind an irregularly shaped, intensely osmiophil body which they consider as presumably lipoidal. They consider it possible that the residual bodies produce in the Sertoli cells some influence which serves to start the changes in the germ-cells and are thus responsible for the local control of spermatogenesis.

The chromatoid body degenerates and disappears in the spermatid of the spider, *Lycosa* (Bösenberg, 1905), and this is also its fate in those spermatids of the crayfish, *Cambarus* (Fasten, 1914), and the grasshopper, *Rhomaleum* (Plough, 1917), in which it occurs.

In the reptiles, *Natrix*, *Lissemys*, and *Uromastix*, the chromatoid bodies remain inactive throughout their existence, not giving any visible proof of their participation in the formation of the spermatozoon (Sud, 1955, 1956, 1957, 1958). During the late stages of spermateleosis they disappear. In all the above-mentioned cases the chromatoid bodies do not appear to contribute anything to the final make-up of the spermatozoon.

Moore (1893, 1894) found that the 'chromatic body' in the spermatid of the rat, formed by the aggregation of the chromatic particles extruded from the nucleus, separates into two unequal parts which are reabsorbed into the nucleus. The Schreiners (1908) state that the chromatoid body in the spermatid of *Myxine* passes into the nucleus and becomes divided into very small particles which appear to play a role in the condensation of the nuclear substance.

Van Molle (1906) observed the chromatoid body in the spermatid of the squirrel constantly near the lower edge of the *manchette*, from the moment the latter appears. It gets further and further away from the nucleus as the *manchette* extends posteriorly and simultaneously fragments into granules which lose themselves in the granulations formed during the regression of the cytoplasm. Van Molle concludes that the proximity of the chromatoid body to the *manchette* from the moment of the appearance of the latter seems to indicate that it participates in its differentiation.

Tobias (1956) in the gerbil, *Tatera brantsii*, found that the chromatoid bodies in the spermatid after fusion into a single body gives rise to the neck-rod, establishes contact with the ring centriole, and is plastered on to the latter. He also noticed an apparent proximity of the fused chromatoid bodies to the developing *manchette*, but is doubtful whether this indicates a casual relation.

Wilson (1925) states that if the chromatoid body has any function this must be performed not later than the spermatocyte divisions, since in many cases it is known to enter only certain of the spermatids. He believes that it may be no more than a by-product of other activities and thus a functionless excretion which does not contribute directly to sperm formation.

In the reptiles (Sud, 1955, 1956, 1957, 1961 *a*, *b*) the chromatoid body reappears during spermateleosis at the time of condensation and elongation of the nucleus, when the histones of chromatin are believed to be replaced by protamines. The chromatoid body, like the sperm nucleus, is characterized by the presence of basic proteins rich in arginine but poor in histones. In addition to proteins the chromatoid body contains much RNA and this may perhaps indicate that it acts as a centre for protein synthesis. On the basis of these conclusions it may be tentatively suggested that the function of this body is to provide basic proteins for the final maturation of chromatin in the nucleus of late spermatids (Sud, 1961 *a*, *b*).

THE SUPPOSED CHROMATOID BODY IN THE SPERMATOGENESIS OF PLANTS

During spermatogenesis of certain liverworts (Schottländer, 1892; Ikeno, 1903; Bolleter, 1905; Humphrey, 1906), mosses (Arens, 1907, the van Leeuwen-Reijnvaans, 1908; Wilson, 1911; Allen, 1917), and a gymnosperm (Hirasé, 1898), prominent bodies of unknown significance have been described. In appearance they resemble chromatoid bodies. They were first referred to as *die chromatoiden Nebenkörper* by Ikeno (1903) in the liverwort, *Marchantia polymorpha*. Ikeno gave the same name to the body previously seen by Schottländer (1892) in the same species. Schottländer figured this body stained red after Rosen's staining method (acid fuchsine followed by methylene blue). In harmony with Ikeno, Bolleter (1905) and Humphrey (1906) in the liverworts, *Fegatella conica* and *Fossombronia longiseta*, and Arens (1907, 1908) and the van Leeuwen-Reijnvaans (1908) in the mosses, *Mnium hornum* and *Polytrichum*, used the term *Nebenkörper*. It seems probable that the two *corps sphériques* in the *Körperzelle* (first generative cell) of the gymnosperm, *Ginkgo biloba* (Hirasé, 1898), which at the time of division are distributed one to each daughter cell (*anthérozoïde* or spermatozooid), are homologous to *der chromatoide Nebenkörper* of Bryophyta. Ikeno (1903) and Allen (1917) also suggested that the *corps sphériques* of Hirasé were probably related to *der chromatoide Nebenkörper*.

Allen (1917) believed that *der chromatoide Nebenkörper* described in plants is different from the one described in the spermatids of certain animals, and he therefore called it 'limosphere', a term first used by Wilson (1911) for this inclusion in plant cells. Though the structures described as *der chromatoide Nebenkörper*, 'limosphere', or *corps sphérique* may be homologous with one another, yet they are chemically different from the chromatoid body of the spermatogenetic cells of animals. Unlike the latter the supposed chromatoid body of plants is not resistant to various fixatives. It is destroyed by fixation in a saturated solution of mercuric chloride in alcohol, in absolute alcohol, in 70% alcohol, or in Merkel's fixative (Hirasé, 1898) Further, whereas the chromatoid body of animals is always deeply stained blue-black by iron haematoxylin, the *corps sphérique* is reported to be stained yellowish brown in the gymnosperm, *Ginkgo* (Hirasé, 1898). (The 'limosphere' in the moss, *Polytrichum*, does stain strongly with iron haematoxylin, though not in early stages (Allen, 1917).) After Rosen's staining method the chromatoid body in the snake, *Natrix natrix*, and in the white rat stain pale blue by the basic dye (Sud, unpublished), but in plants (Schottländer, 1892; Hirasé, 1898) the supposedly homologous body is stained red by the acid dye.

CONCLUSION

From what has been said it may be felt that the descriptions of the morphology, physical and chemical nature, behaviour during division stages, origin, and fate of the chromatoid bodies in different species or even in the same species, as given by different authors, are in many cases most diverse. This has led prominent workers, among them Duesberg (1908), the Schreiners (1908), Wilson (1925), and Gresson (1951), to doubt whether all the bodies named as such are of the same nature. In spite of numerous studies it has not been possible to formulate criteria for the identification of this body from the morphological, chemical, and physiological standpoint. The study of morphology and behaviour with which practically the entire literature is concerned has failed to cater for this need. Now the only hope lies in the chemical analysis of this enigmatic body in different animal groups.

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APPENDIX

The occurrence of the chromatoid body

An entry in the form of a line indicates that the author concerned is certain of his or her observation and names the object chromatoid body, chromatic body, *der chromatoide Nebenkörper*, *der chromatoide Körper*, or *corps chromatoïde*. Dashes mean that the chromatoid body is not invariably present at the stage indicated; continuous dots mean that the author is not certain of the observation or the body is equivocally named *Knöpfchen* (ref. 2, page 292), or wrongly named acroblast (ref. 45), or provisionally named chromatoid body (ref. 35), or only figured and neither named nor mentioned in the text (refs. 1, 30), or is not present as a separate entity (refs. 4, 5, 13, 17, 54). An interrupted row of dots indicates that the author is not sure of the observation, and that the body is not always present at the stage indicated.

		Spermatogonium	*Primary spermatocyte*	*Secondary spermatocyte*	*Spermatid*	*Spermatozoon*	*Ref.**
ARACHNIDA	Opisthothelae						
	Lycosa sp.				- - - - -		12
	Pardosa sp.		——	- - - -			51
CRUSTACEA	Macrura						
	Cambarus virilis		——	- - - -	- - - -		23
	Cambarus virilis		——	- - - -	- - - -		41
	Potamobius sp.		—	— —	—······		34
	Brachyura						
	Cancer magister		——	——	- - -		27
INSECTA	Thysanura						
	Lepisma domestica				·········		31
	Lepisma domestica				——		32
	Orthoptera						
	Rhomaleum micropterum		——	- - - -	- - - -		25
	Scyllina cyanipes		——	- - - -	- - - -		40
	Grasshopper				——		56
	Nemobius sp.		——	——	——		58
	Hemiptera-Heteroptera						
	Pentatoma (*Rhytodolomia*) *senilis*		——	- - - -	- - - -		22, 42
	Pentatoma (*Chlorochroa*) *juniperina*		——	- - - -	- - - -		22
	Podisus crocatus		——	- - - -	- - - -		22
	Caenus delius		- -	- - - -	- - - - -		22
	Gerris remigis			········	·········		35
	Gerris marginatus			········	·········		35
	Coleoptera						
	Dytiscus sp.		——		——		15
	Chelymorpha cassidea				——		31
CYCLOSTOMATA	Myxinoidea						
	Myxine glutinosa		——	——	——	·········	13, 17
	Bdellostoma burgeri		——	——			17
REPTILIA	Testudinata						
	Lissemys p. punctata	——·	··——	——	——		52
	Sauria						
	Uromastix hardwickii	——·	··——··	·——··	· ——		55
	Lizards				·····		30
	Ophidia						
	Natrix p. piscator		——·	··	——		50
	Natrix natrix		——	——	——		59, 60
	Thamnophis sp.				······		45
AVES	Anseres						
	Domestic duck	——	——	——	——		49
MAMMALIA	Metatheria						
	Phalangista vulpina				······		11
	Dasyurus sp., *Sarcophilus* sp. and *Phascolarctus* sp.	·········					37
	Pseudochirus sp., and *Trichosurus* sp.		—	— —	—		37
	Insectivora						
	Hedgehog		——	——	——		3
	Talpa europea		—	— —	—		38
	Primates						
	Man	——	——	——	——		21
	Rodentia						
	White rat				·····		1

		Spermatogonium	Primary spermatocyte	Secondary spermatocyte	Spermatid	Spermatozoon	Ref.*
MAMMALIA	*Rattus* ('*Mus*') *decumanus* var. *albinos*			——	——		16, 18
	Rattus ('*Mus*') *norwegicus albinus*		—	— ——	……		26
	Rattus norvegicus albinoticus		·· ··	· ·· —	——		36
	Albino rat				——		46
	Rat		——	——	——		3
	Rat			——	——	………	4, 5
	Rat		——		——		6
	Rat		——	——	——		7
	Brown rat			——			8
	Rat		——	——	——		19
	Rattus ('*Mus*') *rattus*		——	——	——		29
	Rat				………		44
	Rat				——		48
	Rat				——		55
	Mouse		………				2
	Mouse		——	——	——		3
	Mouse				——		6
	Tatera brantsii draco		——	——	——	………	54
	Guinea-pig		——	——	——		3
	Guinea-pig		——		——		6
	Guinea-pig			——	——		9
	Guinea-pig			——	——		20
	Cavia		——	——	——		28
	Cavia		——	——	——		29
	Guinea-pig				——		33
	Guinea-pig				——		57
	Squirrel		··· ··· ··	——	——		14
	Grey squirrel		——	– – – –	– – – –		39
	Carnivora						
	Putorius furo		—	— —	—		38
	Felis domestica				——		47
	Cat				——		5
	Dog				——		5
	Vulpes fulva		——	– – – –	– – – – –		43
	Perissodactyla						
	Horse		——	– – – –	– – – – –		24
	Artiodactyla						
	Pig		—	—			24
	Bull		——	——			10

* References: 1, Brunn (1876); 2, Hermann (1889); 3, Benda (1891); 4, Moore (1893); 5, Moore (1894); 6, Niessing (1897); 7, Lenhossék (1898); 8, Ebner (1899); 9, Meves (1899); 10, Schœnfeld (1900); 11, Korff (1902); 12, Bösenberg (1905); 13, the Schreiners (1905); 14, Molle (1906); 15, Schäfer (1907); 16, Duesberg (1908); 17, the Schreiners (1908); 18, Duesberg (1909); 19, Regaud (1910); 20, Duesberg (1911); 21, Winiwarter (1912); 22, Wilson (1913); 23, Fasten (1914); 24, Wodsedalek (1914); 25, Plough (1917); 26, Allen (1918); 27, Fasten (1918); 28, Gatenby and Woodger (1921); 29, Ludford and Gatenby (1921); 30, Painter (1921); 31, Bowen (1924); 32, Gatenby and Mukerji (1929); 33, Gatenby and Wigoder (1929); 34, Grabowska (1929); 35, Pollister (1930); 36, Koller and Darlington (1934); 37, Koller (1936*a*); 38, Koller (1936*b*); 39, Koller (1936*c*); 40, Long (1940); 41, McCroan (1940); 42, Schrader (1940); 43, Bishop (1942); 44, Austin and Sapsford (1951); 45, Fox (1952); 46, Watson (1952); 47, Burgos and Fawcett (1955); 48, Daoust and Clermont (1955); 49, Gupta (1955); 50, Sud (1955); 51, Sharma and Gupta (1956); 52, Sud (1956); 53, Swift; (1956); 54, Tobias (1956); 55, Sud (1957); 56, Fawcett and Ito (1958); 57, Gatenby, Tahmisian, Devine, and Beams (1958); 58, Gatenby and Tahmisian (1959); 59, Sud (1961*a*); 60, Sud (1961*b*).

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